CHANGES IN *IN VITRO* PROTEIN-SYNTHESIZING ACTIVITY OF EMBRYONIC FOWL LIVER

By

MON-LI HSIUNG CHU

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TABLE OF CONTENTS

	Page
Acknowledgements	ii
List of Tables	iv
List of Figures	v
Abstract	vii
Introduction	1
Mechanism and Control of Protein Synthesis	1
Liver Protein Synthesis	6
Research Objectives	11
Materials and Methods	13
Materials	13
Biological Preparations	14
Biochemical Determinations	22
Results and Discussion	30
Characteristics of the Cell-Free Protein-Synthesizing System Derived From Embryo Livers at Various Stages of Development	30
Distribution and Activity of Free and Membrane-Bound Polysomes	62
Albumin Synthesis	79
Conclusions	93
Bibliography	96
Biographical Sketch	101

LIST OF TABLES

Table		Page
1	Dependence of protein synthesis on various components of the reaction mixture	32
2	Subcellular distribution of Aminoacyl-tRNA synthetase activity	53
3	Amino acid requirement of the pyrophosphate-ATP exchange reaction	54
4	Ribonuclease activity in liver subcellular fractions derived from embryos of various ages	59
5	Ribonuclease inhibitor activity in cell sap derived from embryos of various ages.	61
6	Distribution of free and membrane-bound polysomes in homogenates of 10-day and 17-day mixture	66
7	Amino acid incorporating activity of free and membrane-bound polysomes before and after Triton X-100 treatment	68
8	Ribonuclease activity in free and membrane-bound polysomes	77
9	Albumin synthesis by membrane-bound and free liver polysomes derived from embryos of various ages.	87
10	Albumin synthesis by 3000 X g supernatant derived from embryos of various ages.	92

LIST OF FIGURES

Figure		Page
1	Preparation of total and salt-washed polysomes.	15
2	Preparation of total cytoplasmic and free polysomes from postnuclear supernatant.	17
3	Preparation of free and membrane-bound polysomes from postmitochondrial supernatant.	19
4	Effect of cell sap concentration on the [^{3}H] lysine incorporation into protein.	33
5	Effect of polysome concentration on the [^{3}H] lysine incorporation into protein.	34
6	Time course of [^{3}H] lysine incorporation into protein . . .	35
7	Amino acid incorporating activity of cell-free systems derived from 12-day and 19-day embryos.	36
8	Amino acid incorporation by total polysomes derived from embryos at various stages of development	38
9	Sucrose gradient profiles of total polysomes derived from various age embryos.	40
10	Free amino acid content of the cell sap derived from embryos at various stages of developement.	43
11	Lysine profiles from amino acid analysis.	44
12	Amino acid incorporating activity of cell saps derived from embryos of various ages.	46
13	Cell sap dependence of [^{3}H] lysine incorporation.	47
14	Time course of incorporation of [^{3}H] lysine from [^{3}H] lysyl-tRNA into polypeptide.	49
15	Effect of cell sap concentration on the incorporation of [^{3}H] lysyl-tRNA into polypeptide	50
16	Lysine incorporation from [^{3}H] lysyl-tRNA into polypeptide by cell saps derived from embryos of various ages	51
17	Time course of pyrophosphate-ATP exchange assay	55

18	Effect of cell sap concentration on the pyrophosphate exchange reaction	56
19	Aminoacyl-tRNA synthetase activity of cell sap derived from embryos of various ages.	58
20	Distribution of free and membrane-bound polysomes in livers at various stages of development	64
21	Sucrose gradient profiles of membrane-bound and free polysomes prepared from 19-day embryonic livers without using detergent.	70
22	Time courses of amino acid incorporation of membrane-bound and free polysomes	72
23	Sucrose gradient profiles of membrane-bound polysomes after Triton X-100 treatment.	74
24	Sucrose gradient profiles of free polysomes after Triton X-100 treatment.	76
25	Polyacrylamide gel electrophoresis of cell-free protein products released from membrane-bound and free polysomes before sonication.	81
26	Polyacrylamide gel electrophoresis of cell-free reaction products released from membrane-bound and free polysomes by sonication.	83
27	Polyacrylamide gel electrophoresis of cell-free protein products of 16-day membrane-bound polysomes remaining after precipitation with antiserum against albumin	84
28	SDS-polyacrylamide gel electrophoresis of the cell-free protein products precipitated with antiserum against albumin	85
29	Polyacrylamide gel electrophoresis of cell-free protein products synthesized by 3,000 X g supernatant from livers at various stages of development	90

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By

Mon-Li Hsiung Chu

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At selected times during chick embryo liver development, a cell-free protein synthesizing system composed of polysomes and cell sap was prepared. The amino acid incorporating activity of cell sap decreased with increasing developmental age when assayed using standard polysomes. Polysomes prepared from embryos of different ages were uniformly active in amino acid incorporation when assayed with standard cell sap. Cell sap aminoacyl-tRNA synthetase activity remained constant during development, whereas the activity in transferring amino acid from aminoacyl-tRNA into polypeptide decreased with developmental age. Ribonuclease activity in the embryonic liver increased rapidly with age. The decline in amino acid incorporating activity of the cell sap is ascribed to the lack of a cell sap component involved in a stage of protein synthesis subsequent to the aminoacylation of the tRNAs or to increasing ribonuclease activity, or both.

As development progressed, the free polysome content of embryonic liver decreased, while membrane-bound polysome content increased markedly. Total polysome content remained relatively constant during development.

The increase in membrane-bound polysome content correlates with and may be responsible for the increase in the secretion of serum proteins by liver cells during development. Membrane-bound polysomes were less active in amino acid incorporation than free polysomes derived from embryos of the same age and this activity difference was more pronounced in older embryos. The activity of free polysomes remained constant with age. High ribonuclease activity was found in the membrane-bound polysome preparation. The difference in amino acid incorporating activity and ribonuclease activity between free and membrane-bound polysomes was abolished after polysomes were treated with Triton X-100. Ribonuclease activity present in the membrane, therefore, probably inhibits the amino acid incorporating activity of membrane-bound polysomes accumulating upon maturation of the embryo.

Albumin synthesis in the cell-free system was detected by polyacrylamide gel electrophoresis and immunoprecipitation. Membrane-bound polysomes were found to be the major site for albumin synthesis in embryonic liver cells. The percentage of albumin in total protein synthesized by membrane-bound polysomes derived from embryos of different ages was relatively constant. Measurement of total protein synthesis by the 3,000 X g supernatant of liver homogenate showed that the percentage of albumin synthesis increased in parallel with developmental age. Correlations between these results and the differentiation of serum proteins are discussed.

INTRODUCTION

Mechanism and Control of Protein Synthesis

Spectacular progress in our knowledge of the biochemistry of protein synthesis has occurred in the past two decades. Much of our present understanding of the mechanism and control of protein synthesis has been obtained from studies in cell-free systems. The first such system was prepared from rat liver (Siekevitz and Zamecnik, 1951; Siekevitz, 1952; Zamecnik and Keller, 1954). Since then, cell-free protein-synthesizing systems have been prepared from a wide range of different organisms. Among them are the rabbit reticulocyte system, first studied by Schweet *et al.* (1958), and the *E. coli* system, first studied by Lamborg and Zamecnik (1960). From studies on these systems, it is now known that protein synthesis takes place on ribosomes; that enzymes and factors present in the high speed supernatant of cell homogenate (cell sap) are required for protein synthesis; that messenger RNA (mRNA) provides the code for amino acid sequence of each protein; that ribosomes move along the mRNA as protein synthesis proceeds. Since several ribosomes can be accommodated on a single mRNA molecule, polyribosomes (polysomes) are formed. The presence of polysomes indicates that cells are actively synthesizing proteins. The events involved in protein synthesis may be divided into four stages: activation of amino acids, polypeptide chain initiation, chain elongation and chain termination.

The first step in protein synthesis is the ATP-dependent activation of amino acids by a class of enzymes known as aminoacyl-tRNA synthetases, each of which is specific for one amino acid (Hoagland, 1955; Hoagland, Keller and Zamecnik, 1956). The activation reaction occurs in two separate steps with the formation of aminoacyl-adenylate as an intermediate (Hoagland *et al.*, 1957).

$$\text{Amino acid} + \text{ATP} + \text{Synthetase} \overset{Mg^{++}}{\rightleftharpoons} (\text{aminoacyl - AMP} + \text{Synthetase}) + \text{PPi} \quad (1)$$

$$(\text{Aminoacyl - AMP} + \text{Synthetase}) + \text{tRNA} \overset{Mg^{++}}{\rightleftharpoons} \text{Aminoacyl - tRNA} + \text{AMP} + \text{Synthetase} \quad (2)$$

$$\text{Amino acid} + \text{tRNA} \overset{\text{Synthetase}}{\rightleftharpoons} \text{Aminoacyl - tRNA} + \text{AMP} + \text{PPi} \quad (3)$$

The activation reaction is usually assayed by measuring the amino acid-dependent rate at which ^{32}P labeled pyrophosphate is incorporated into added ATP in Reaction (1) catalyzed by the synthetases (Berg, 1956).

Initiation of protein synthesis begins with the binding of initiating aminoacyl-tRNA and mRNA to the small subunit of the ribosome in the presence of GTP and initiation factors to form the "initiation complex," which then binds to the large subunit of the ribosome to form a complete ribosome (Haselkorn and Rothman-Denes, 1973). Initiation factors can extracted from the small ribosome subunit with 0.5 to 2.0 M NH_4Cl KCl (Shafritz *et al.*, 1970; Heywood and Thompson, 1971; Kaempfer and Kaufman, 1972).

Elongation of polypeptide chain can be considered as a three-step process (Haselkorn and Rothman-Denes, 1973). The first step is the binding of the incoming aminoacyl-tRNA to the acceptor site (A site) of the ribosome. This binding requires GTP and an elongation factor termed EF-T in prokaryotes and EF-1 in eukaryotes. The second phase is the

formation of a peptide bond linking the amino group of the aminoacyl-tRNA with the peptide moiety of the peptidyl-tRNA, located at the peptidyl site (P site) of the ribosome, to form a new peptidyl-tRNA, one amino acid unit longer. This step is catalyzed by the enzyme, peptidyl transferase, which is an integral part of the large ribosome subunit (Monro, 1967). The third step is the translocation of the new peptidyl-tRNA from the A site to the P site of the ribosome, thus freeing the A site for the next aminoacyl-tRNA (Traut and Monro, 1964). The translocation process requires GTP and another elongation factor, termed EF-G in prokaryotes and EF-2 in eukaryotes.

Polypeptide chain termination is signaled by termination codons (UAA, UAG and UGA) in the mRNA. When a termination codon is reached, the ribosome binds a release factor, R_1 or R_2, which activates peptidyl transferase, which then hydrolyzes the ester linkage between polypeptide and tRNA (Caskey *et al.*, 1969; Capecchi and Klein, 1969).

Changes in the protein synthetic capacity of cells have been reported to occur in response to altered nutritional conditions, hormone stimulation and various pathological states (Pain and Clemens, 1973). These alterations of protein synthetic activity are often ascribed to variations in ribosomal and/or supernatant activities.

Several mechanisms could operate to alter the protein synthetic activity of ribosomes. The rate of protein synthesis depends on the number of ribosomes per cell, the proportion of ribosomes associated with mRNA and the protein-synthesizing activity of polysomes (Monro *et al.*, 1953; Wilson and Hoagland, 1967).

Current evidence suggests that chain initiation is the rate limiting step for translation in most cells. The number of active

ribosomes found in polysomes is regulated by the initiation process (Kaempfer, 1971). Messenger-specific initiation factors, capable of discriminating between classes of mRNAs, have been implicated in the regulation of protein synthesis in eukaryotes. Heywood (1970) demonstrated that initiation factors removed from chick muscle ribosomes by a high salt wash were required for both the binding of muscle mRNA to ribosomes and the mRNA-directed synthesis of myosin on reticulocyte ribosomes. Ilan and Ilan (1971) reported that, during insect development, there was a stage-specific initiation factor which promoted the formation of the complete initiation complex only with mRNA extracted from the same stage of development. However, other workers have found no requirement for specific factors for translation of mRNA in heterologous system (Lane *et al.*, 1971; Rhoads *et al.*, 1971; Sampson *et al.*, 1972).

Elongation factors, EF-1 and EF-2, are present both free in cell sap and bound to ribosomes. Those factors, when bound to ribosomes, may influence the activity of ribosomes in a cell-free system. Alexis *et al.* (1972) found that crude muscle ribosomes from protein-deficient rats showed lower protein-synthesizing activity than ribosomes from normal rats. The activity difference between these two kinds of muscle ribosomes were related to a difference in the content and/or activity of non-ribosomal factors associated with the ribosome preparations rather than to alterations in the ribosomes themselves. An increase in EF-1 activity in the spleen of rats was observed following immunization (Willis and Starr, 1971). In HeLa cells, EF-2 content increased during rapid growth and decreased when growth slowed (Hendriksen and Samlson, 1972). In addition, much of the EF-2 was present in the cell sap when the growth rate was high,

while a large proportion of EF-2 was associated with 80 S ribosomes when protein synthesis was restricted.

Aminoacyl-tRNA synthetases regulate the rate of translation in several different ways. First, the activity of the synthetases varies with the rate of general protein synthesis in various physiological states. Diabetes, which lowers the rate of protein synthesis in rat muscle, also lowers the activity of synthetases in muscle cell sap (Pain, 1971). Secondly, changes in the pattern of protein synthesis during growth and development are often accompanied by changes in the spectrum of activity of synthetases specific for different amino acids. The synthesis of phosvitin, a serine-rich egg yolk protein, by the liver of laying hens was accompanied by an increase in seryl-tRNA synthetase level (Beck, Hentzen and Ebel, 1970). Thirdly, during protein or amino acid deficiency, synthetase activity rises, facilitating amino acid conversion into protein. One example of such a phenomenon is that protein deficiency in the rat results in increased activity of hepatic aminoacyl-tRNA synthetases (Mariani, Spadoni and Tomassi, 1963).

The level of ribonuclease and ribonuclease inhibitor activities has been shown to vary in different metabolic states of the cell. Kraft and Shortman (1970) suggested that high inhibitor/ribonuclease ratios were associated with the state of cytoplasmic RNA accumulation while low ratios were associated with net RNA catabolism. The level of cellular RNA, especially that of mRNA, controls the rate of protein synthesis. A decrease in ribonuclease activity (Arora and de Lamirande, 1967) and an increase in ribonuclease inhibitor activity (Shortman, 1962; Moriyama *et al.*, 1969) have been noted in rats following partial hepatectomy. Protein deficiency in the rat resulted in lower ribo-

nuclease inhibitor activity and higher ribonuclease activity in the liver cell as compared with well-fed control rats (Sheppard *et al.*, 1970). The low amino acid-incorporating activity of cell-free systems derived from chicken liver was ascribed to an uninhibited ribonuclease in the chicken liver cell sap (Siler and Fried, 1968). Burka (1970) found a ribonuclease activity in the reticulocyte cell-free system which was operating during the period when protein synthesis occurred but was not active at 0°C.

Liver Protein Synthesis

The liver's special function of secreting considerable amounts of proteins into blood plasma makes this tissue particularly attractive to those interested in studying the mechanism of regulation of protein synthesis. In addition, the liver is a large discrete organ and is relatively easy to fractionate into subcellular components. Albumin is the most abundant of all the proteins found in the serum of most vertebrates, representing 40-50% of the total plasma proteins (Engle and Woods, 1960). Some of the early studies on albumin synthesis were carried out in chicken liver. Peters and Anfinsen (1950) demonstrated a net synthesis of serum albumin when slices of chicken liver were incubated with $^{14}CO_2$. Peters (1959) studied the intracellular distribution of the newly synthesized albumin. He found that if the chicken liver slices were disrupted and fractionated, some albumin was released from both mitochondrial and microsomal fractions by deoxycholate treatment. Of these two fractions, microsomal albumin was found to be more radioactive. He tested the effectiveness of various reagents in releasing

serum albumin from the microsomal pellet and found that only those substances which dissolved lipid were effective in releasing serum albumin. Peters concluded that albumin was held in intimate association with the lipid membrane. He also characterized the microsomal albumin with regard to sedimentation rate, electrophoretic mobility and N-terminal and C-terminal amino acid residues (Peters *et al.*, 1958). Continuation of such studies in the cell-free system was unsuccessful, because the microsome fraction isolated from chicken liver was relatively inactive in amino acid incorporation as compared with that from rat liver (Campbell and Kernot, 1959). Therefore, the rat liver has been used as a model system for studying albumin synthesis by most investigators.

In protein-secreting cells such as liver cells, some polysomes are bound to the membranes of the endoplasmic reticulum and some polysomes are free in the cytoplasm (Palade and Siekevitz, 1956). Membrane-bound polysomes are thought to be engaged in making those proteins secreted by the cell, whereas free polysomes synthesize proteins that are to be retained within the cell (Siekevitz and Palade, 1960; Vassalli, 1967). Redman (1968) first demonstrated that membrane-bound polysomes were the main or exclusive site of albumin synthesis in rat liver cells. Takagi and Ogata (1968) made similar findings. Hicks *et al.* (1969) compared the efficiency of free polysomes and total (free and membrane-bound) polysomes of rat liver cells in making ferritin, a retained protein, and albumin in a cell-free system. They showed that the total polysome fraction was more efficient for albumin synthesis, presumably because it contained membrane-bound polysomes. On the other hand, free polysomes

were more efficient in ferritin synthesis. Uenoyama and Ono (1972) reported that albumin was synthesized by free polysomes from 5123 hepatoma, a system in which the synthesized albumin is not secreted but retained in the cell. Taylor and Schimke (1973) demonstrated that rat liver polysomal RNA was capable of directing albumin synthesis in a cell-free system derived from rabbit reticulocytes. The RNA extracted from membrane-bound and free polysomes of rabbit liver has been shown to direct albumin and ferritin synthesis in a reticulocyte cell-free system (Shafritz *et al.*, 1973; Shafritz, 1974a).

Albumin synthesis during embryonic development is an interesting subject for studying the control of protein synthesis. The chick embryo is an ideal organism for such study, because it is relatively easy to obtain in large quantities and the embryonic blood is separate from the maternal circulation. The blood proteins of the chick embryo, especially in early stages of development, are very different from the pattern characteristic of adult chicken. There is an increase in the number of serum proteins as well as an increase in the concentration of most components with age (Weller and Schechtman, 1962). Albumin first appears as a distinct component in 9-day serum, its concentration increasing from 13% of total serum proteins at the 9th day of development up to 50% at the 19th day and then showing a slight decrease by hatching time on the 21st day. The differentiation of serum proteins from embryonic to adult type is probably a direct result of functional differentiation of the liver cells in their increasing ability to synthesize adult proteins.

The chick liver appears at the end of the 2nd day of embryonic life. It weighs approximately 2 mg on the 5th day, 50 mg on the 10th

day and 1 g at hatching time (Romanoff, 1967, p. 68). The growth of the liver, expressed as a percentage of its hatching weight, when plotted against incubation time, gives a characteristic sigmoid-shaped curve. However, when the growth rate of liver is plotted against time, there is a rapid fall from very high to low values between the 7th and 9th day of incubation. Thereafter, the growth rate decreases gradually until hatching time (Romanoff, 1967, p. 267). It is believed that the decrease in the specific rate of growth is due to the slowing down of metabolism caused by the reduction in the rate at which the tissues are supplied with nutrients (Byerly, 1932). Early embryonic liver cells lack many ultrastructural features of adult liver cells (Pollak and Shorey, 1967). Electron micrographs of 5-day embryonic liver cells show that there is practically no endoplasmic reticulum present in the cytoplasm. The amount of endoplasmic reticulum increases as development progresses, with smooth endoplasmic reticulum appearing prior to rough endoplasmic reticulum.

There is a considerable amount of information about variations in enzyme patterns during chick liver development. In 1967, Romanoff (p. 106) listed 23 enzymes that have been studied in the embryonic liver. Recently, Greengard and Thorndike (1974) reviewed studies on 52 enzymes in the same organ. They classified enzymes according to the time of their emergence. The majority of enzymes emerge during embryonic development. Some enzymes attain their mature level at the earliest time test (about 10th day of incubation), while others are still absent at the time of hatching. It is now well established that each enzyme increases at its own rate, according to its own pattern.

The appearance of new enzymes results in new metabolic potentialities. However, it is sometimes difficult to interpret the physiological role of quantitative changes in enzyme levels.

Protein or albumin synthesis in chick embryos has been previously studied in liver slices or *in vivo*. Duck-Chong *et al.* (1964) studied the protein-synthesizing activity of chicken liver by incubating liver slices of 8-, 14- and 20-day embryos and adult chicken with [^{14}C] leucine. They found that embryonic liver slices incorporated [^{14}C] leucine 15 to 30 times more rapidly than adult liver slices. In the three different age embryos they studied, the rate of incorporation was most rapid at 8-day and decreased as development proceeded. Reade *et al.* (1965) injected [^{14}C] leucine into 14- to 20-day embryos and then isolated various proteins from embryonic serum by electrophoresis. They showed that label was incorporated into the albumin fraction. By immunochemical techniques, Zaccheo and Grossi (1967) observed that serum albumin could be detected in the circulating blood of the embryo at the 4th day of development; however, no albumin was found in the liver microsomal fraction until the 8th day of incubation. The yolk sac is the only other embryonic tissue capable of producing albumin (Butler, 1972); albumin thus appears to be supplied to the embryo from the yolk sac before it is synthesized in the liver.

One approach to understanding the changes involved in the control of protein synthesis has been the study of a cell-free system. As previously mentioned, the microsome fraction isolated from chicken liver was relatively inactive in amino acid incorporation (Campbell and Kernot, 1959). Siler and Fried (1968) studied the poly (U)-

dependent polyphenylalanine synthesis in both homologous and heterologous systems composed of mixtures of ribosomes and cell sap prepared from chicken and rat livers and found that with a given ribosome preparation, chicken liver cell sap was much less active in phenylalanine incorporation than rat liver cell sap. This relative inactivity was ascribed to the presence of an uninhibited nuclease in chicken liver cell sap. Zimmerman and Fried (1971) found that the preparation of active ribosomes from adult and embryonic chicken livers required a higher salt concentration (250 mM KCl) during isolation than was common in the isolation of ribosomes from rat liver (50 mM KCl). Jernigan *et al.* (1972) further studied the effect of KCl concentration on the yield and the polyphenylalanine-synthesizing activity of chicken liver ribosomes. KCl concentrations of 150-250 mM in the isolation medium resulted in higher ribosome yield and activity than 25-50 mM. Polysomal amino acid-incorporating systems have been prepared from chicken liver by Jernigan *et al.* (1973). The high nuclease activity in chicken liver was minimized by the use of bentonite and low molecular weight yeast RNA as nuclease inhibitors. In the presence of such nuclease inhibitors, and under appropriate ionic conditions, high molecular weight polysomes which are active in protein synthesis were obtained. Thus the study of the regulation of serum protein synthesis by components isolated from the developing chicken liver is feasible.

Research Objectives

The purpose of this research is to study specific protein synthesis during chick embryonic liver development with serum albumin

used as a probe for such study. As a first step in achieving this aim the following questions were asked. (1) Does the protein synthesizing activity of the components of a cell-free system derived from the livers of chick embryos change during development? (2) Is there a correlation between the structural differentiation of liver cells with the onset of serum protein synthesis? (3) Can albumin synthesis be measured in the cell-free system derived from embryonic chick livers? (4) Does albumin synthesis measured in the cell-free system correlate with albumin synthesis *in vivo*?

MATERIALS AND METHODS

Materials

Animals

Fertile white Leghorn eggs were obtained from the Poultry Science Department of the University of Florida, and incubated, blunt end up, at 38°C in a Leahy model 624 electric incubator. The eggs were turned three times a day. The age of the embryos was measured from the start of incubation. Under these conditions of incubation, the hatching time was 21 days.

Chemicals

Ribonuclease-free sucrose, [^{3}H] lysine, [^{3}H] leucine were purchased from Schwartz-Mann. ATP, disodium salt; GTP, sodium salt; phosphoenol-pyruvate, monopotassium salt; pyruvate kinase, Type II from rabbit skeletal muscle; bovine pancreatic ribonuclease (Type XII-A); Torula yeast RNA (Type VI); 2,5-diphenyloxazole and 1,4-bis-2(4-methyl-5-phenyloxazole) were purchased from Sigma Chemical Company. ^{32}P labeled sodium pyrophosphate and Aquasol scintillation fluid were purchased from New England Nuclear. Acrylamide and bisacrylamide were purchased from Eastman Kodak Company. The more common organic or inorganic reagents were analytical or reagent grade.

Biological Preparations

Preparation of Total Polysomes

Chick livers from embryos of the desired age were dissected out and immersed in ice cold Buffer I (150 mM KCl, 8 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4) containing 250 mM sucrose. The pooled livers were blotted dry with paper towel, weighed and then homogenized in 3 volumes of ice-cold Buffer I containing 250 mM sucrose, 5 mg/ml bentonite and 78.5 A_{260} units (the absorbance at 260 nm)/ml yeast RNA at 120 rev./min by 4 strokes of a glass-Teflon homogenizer. The homogenate was centrifuged for 15 min at 12,000 X g_{max} in a Beckman-Spinco Model L centrifuge to ensure complete removal of mitochondria (Figure 1). Nineteen parts of this postmitochondrial supernatant were mixed with 1 part of 20% Triton X-100, pH 7.4 and then 3 ml of this suspension were layered over a discontinuous gradient of 3 ml and 2 ml of Buffer I containing 0.5 M and 2.0 M sucrose respectively. The tubes containing these gradients were centrifuged for 2.5 hours at 226,000 X g_{max} in a Spinco Model L centrifuge. The pellets were rinsed several times with Buffer I and then resuspended in 0.25 ml Buffer I/ml original postmitochondrial supernatant. The suspensions were then clarified by centrifugation at 12,000 X g_{max} for 10 min and used immediately for amino acid incorporation studies. The concentration of soluble polysomes in such preparations was about 20 A_{260} units/ml. The A_{260}/A_{280} ratio of such polysome preparations was in the range 1.77-1.87.

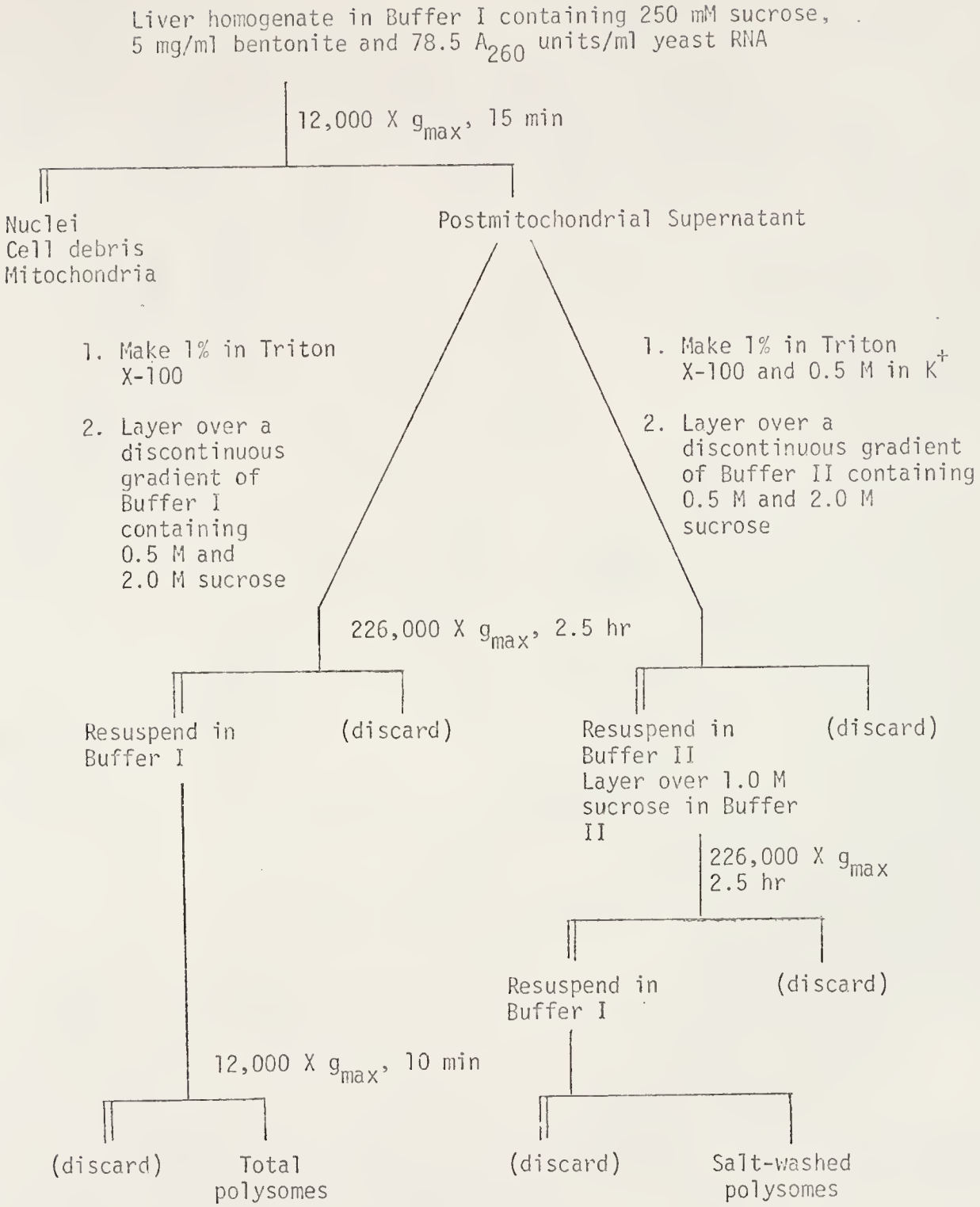

Liver homogenate in Buffer I containing 250 mM sucrose, 5 mg/ml bentonite and 78.5 A_{260} units/ml yeast RNA
12,000 X g_{max}, 15 min
Nuclei
Cell debris
Mitochondria
Postmitochondrial Supernatant
1. Make 1% in Triton X-100
2. Layer over a discontinuous gradient of Buffer I containing 0.5 M and 2.0 M sucrose
1. Make 1% in Triton X-100 and 0.5 M in K^+
2. Layer over a discontinuous gradient of Buffer II containing 0.5 M and 2.0 M sucrose
226,000 X g_{max}, 2.5 hr
Resuspend in Buffer I
(discard)
Resuspend in Buffer II
Layer over 1.0 M sucrose in Buffer II
(discard)
226,000 X g_{max}
2.5 hr
Resuspend in Buffer I
(discard)
12,000 X g_{max}, 10 min
(discard)
Total polysomes
(discard)
Salt-washed polysomes

Figure 1. Preparation of total and salt-washed polysomes.

Preparation of Salt-Washed Polysomes

The postmitochondrial supernatant prepared as described above was treated with 1/19 volume of 20% Triton X-100 and made 0.5 M in K^+ by the addition of 2.5 M KCl (Figure 1). The mixture was then layered over a discontinuous gradient of 3 ml and 2 ml of Buffer II (500 mM KCl, 8 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4) containing 0.5 M and 2.0 M sucrose respectively. After centrifugation at 226,000 X g_{max} for 2.5 hours, the pellet was rinsed and resuspended in Buffer II. The suspension was then layered over 2 ml of 1.0 M sucrose in Buffer II, and centrifuged again at 226,000 X g_{max} for 2.5 hours. The pellet was resuspended in Buffer I and clarified by centrifugation at 12,000 X g_{max} for 10 min. This suspension is designated "salt-washed polysomes."

Preparation of Free and Membrane-Bound Polysomes from Postnuclear Supernatant

The preparation of free and total cytoplasmic polysomes from postnuclear supernatant followed the procedure described by Blobel and Potter (1967) with some modification (Figure 2). The livers were homogenized in 3 volumes of Buffer I containing 250 mM sucrose, by 15 strokes at 120 rev./min in a glass-Teflon homogenizer. One milliliter of the homogenate was mixed with 2 ml of Buffer I containing 2.2 M sucrose. This was then layered over 1 ml of 2.2 M sucrose in Buffer I. The sample was centrifuged in a Spinco SW 50.1 swinging bucket rotor at 170,000 X g_{max} for 40 min. The supernatant was poured off and mixed with the material adhering to the wall of the tube. This was rehomogenized manually by 4 strokes of a glass-Teflon homogenizer. The resulting solution was

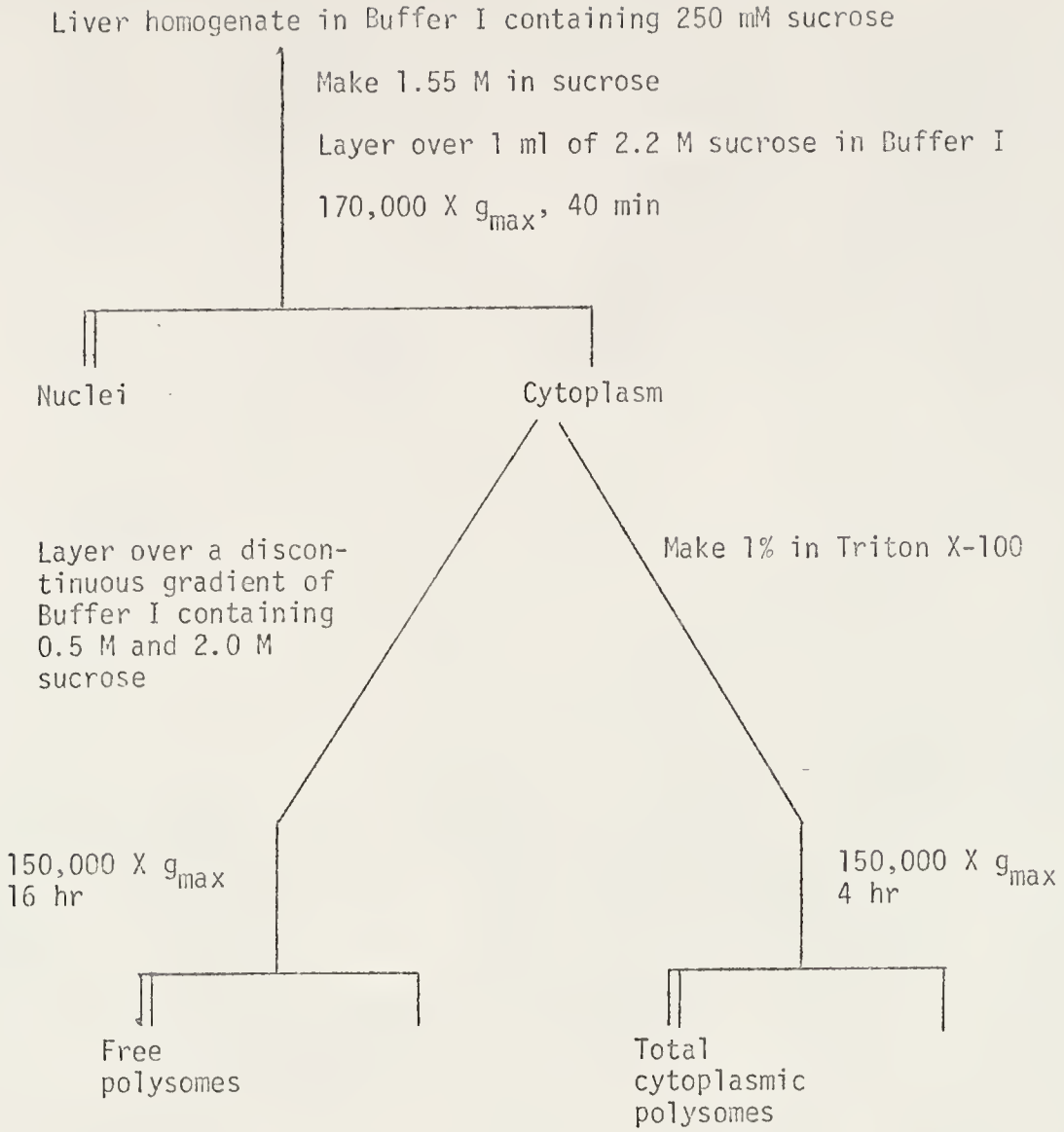

Liver homogenate in Buffer I containing 250 mM sucrose
Make 1.55 M in sucrose
Layer over 1 ml of 2.2 M sucrose in Buffer I
170,000 X g max, 40 min
Nuclei
Cytoplasm
Layer over a discontinuous gradient of Buffer I containing 0.5 M and 2.0 M sucrose
Make 1% in Triton X-100
150,000 X g max 16 hr
150,000 X g max 4 hr
Free polysomes
Total cytoplasmic polysomes

Figure 2. Preparation of total cytoplasmic and free polysomes from postnuclear supernatant.

designated "postnuclear supernatant." For the preparation of free ribosomes, 1 ml of postnuclear supernatant was layered over 3 ml of 2.0 M sucrose in Buffer I and the tube was filled with Buffer I containing 250 mM sucrose. The sample was centrifuged in a Spinco Ti 50 rotor at 150,000 X g_{max} for 16 hours. The pellet contained the free polysomes. For the preparation of total cytoplasmic polysomes, 1 ml of the postnuclear supernatant was mixed with 0.4 ml of 20% Triton X-100. Buffer I was added to it to provide a total volume of 8 ml. The sample was centrifuged in a Spinco Ti 50 rotor at 150,000 X g_{max} for 4 hours. The pellet contained the total cytoplasmic polysomes. Membrane-bound polysomes were determined by measuring the difference between total cytoplasmic and free polysomes.

Preparation of Free and Membrane-Bound Polysomes from Postmitochondrial Supernatant

Polysomes were prepared from postmitochondrial supernatant by a modification of the procedure (Figure 3) described by Blobel and Potter (1967). The postmitochondrial supernatant prepared as described previously was layered over a discontinuous gradient containing 2.0 ml and 3.0 ml of 2.0 M sucrose and 1.35 M sucrose respectively in Buffer I. The gradient was centrifuged at 226,000 X g_{max} for 4 hours. Free polysomes sediment in a pellet at the bottom of the tube whereas the membrane-bound polysomes form a band at the boundary of the two sucrose layers. The membrane-bound polysomes were removed from the top by suction. Free polysomes were resuspended in Buffer I by homogenizing by hand. In some experiments, the free and membrane-bound polysomes prepared under these conditions were treated with 1/19 volume of 20% Triton X-100 and then

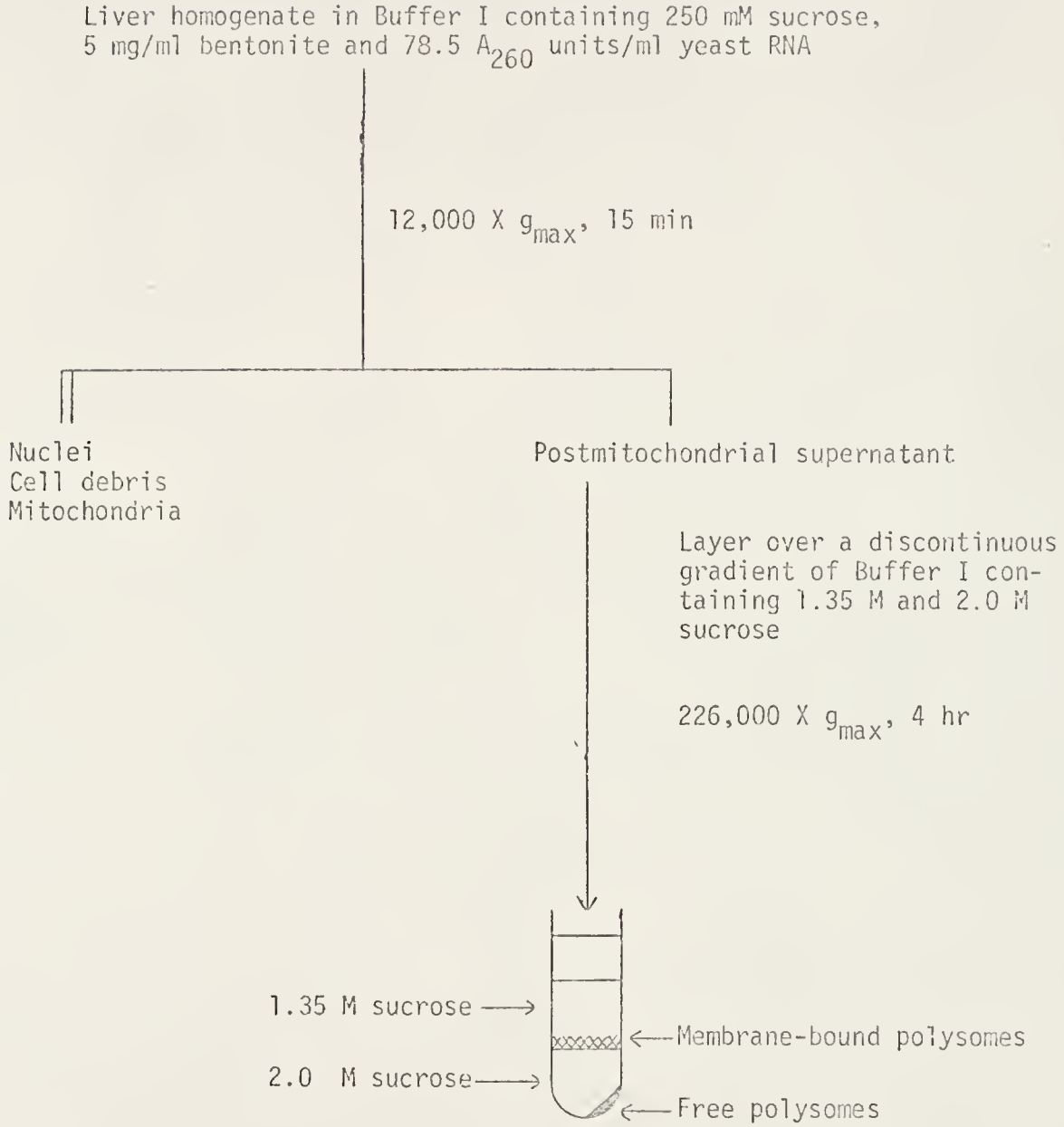

Liver homogenate in Buffer I containing 250 mM sucrose, 5 mg/ml bentonite and 78.5 A_{260} units/ml yeast RNA
12,000 X g_{max}, 15 min
Nuclei
Cell debris
Mitochondria
Postmitochondrial supernatant
Layer over a discontinuous gradient of Buffer I containing 1.35 M and 2.0 M sucrose
226,000 X g_{max}, 4 hr
1.35 M sucrose
Membrane-bound polysomes
2.0 M sucrose
Free polysomes

Figure 3. Preparation of free and membrane-bound polysomes from postmitochondrial supernatant.

layered over 2.0 ml of 1.0 M sucrose in Buffer I. The gradients were centrifuged again in a Spinco Ti 50 rotor at 226,000 X g_{max} for 2.5 hours.

Preparation of Cell Sap and Microsomes

One part of liver was homogenized in 3 to 6 parts of Buffer I containing 250 mM sucrose at 120 rev./min by 10 strokes of a glass-Teflon homogenizer (without bentonite and yeast RNA). The homogenate was centrifuged at 12,000 X g_{max} for 15 min to prepare a postmitochondrial supernatant. Cell sap was prepared by centrifuging the postmitochondrial supernatant at 226,000 X g_{max} for 1.5 hours. The resulting microsomal pellet was resuspended in Buffer I containing 250 mM sucrose.

Preparation of 3,000 X g Supernatant

Livers were homogenized in 3 volumes of Buffer I containing 250 mM sucrose at 120 rev./min by 10 strokes of a glass-Teflon homogenizer. The homogenate was centrifuged at 3,000 X g_{max} for 10 min and the resulting supernatant was removed from the centrifuge tube with a syringe.

Preparation of [^{3}H] lysyl-tRNA

[^{3}H] lysyl-tRNA was prepared from 1-week chick livers by the method of von Ehrenstein (1967). Fifty grams of livers were homogenized in 3 volumes of Buffer I containing 250 mM sucrose and then centrifuged at 12,000 X g_{max} for 15 min. The postmitochondrial supernatant was extracted two times with an equal volume of buffer saturated phenol. Nucleic acids were precipitated from the aqueous phase by adding 0.1 volume of cold

20% potassium acetate (pH 5) and 2.5 volume of -20°C 95% ethanol and held overnight at -20°C. The precipitates were centrifuged and extracted twice with 100 ml cold 1M NaCl. The supernatants from the two extractions were combined and tRNAs were precipitated by adding 2.5 volume of -20°C 95% ethanol.

To discharge the amino acids, the tRNAs were incubated in 20 ml of 0.5 M Tris-HCl buffer (pH 8.8) for 1 hour at 35°C and then reprecipitated by the addition of 0.1 volume of 20% potassium acetate (pH 5) and 2.5 volume of -20°C 95% ethanol.

The tRNAs thus obtained were further purified by dissolving in 10 ml of 0.1 M Tris-HCl buffer, pH 7.5, and applying the solution to a DEAE cellulose column equilibrated with the same buffer. After washing the column with 10 volumes of 0.1 M Tris-HCl buffer, pH 7.5, tRNAs were eluted by 1 M NaCl solution in 0.1 M Tris-HCl, pH 7.5, and then precipitated again by 2.5 volume of -20°C 95% ethanol.

To charge the purified tRNAs with [^{3}H] lysine, a final volume of 5 ml contained: 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM KCl, 2 mM ATP, 1 mM dithioerythritol, 0.5 mCi [^{3}H] lysine (specific activity 10 Ci/mmole), 0.2 mM of 19 other amino acids, cell sap (approximately 3.0 mg of protein from which endogeneous amino acids were removed by passing through a Sephadex G-25 column), and 10 mg of purified tRNAs. Following incubation at 35°C for 20 min, the mixture was chilled in ice and extracted with an equal volume of buffer saturated phenol. Charged tRNAs were precipitated from the aqueous phase as described before. It was then dissolved in H_2O and dialyzed exhaustively against several changes of H_2O. The charged tRNAs were lyophilized and stored at -20°C.

Preparation of Nuclease Inhibitors

Bentonite was prepared by a modification of the procedure described by Fraenkel-Conrat *et al.* (1961). Fifty grams of bentonitewere suspended in 1 liter of a solution containing 8 mM $MgCl_2$ and 2 mM EDTA and then stirred for 3 days. The bentonite was then washed by centrifuging 4 times in 10 mM Tris-HCl, pH 7.4, containing 8 mM $MgCl_2$, each time discarding the portion which sedimented in 5 min at 500 rev./min and the portion which remained in suspension after 10 min at 8,000 rev./min. The final 500 rev./min supernatant suspension (50 mg/ml) was frozen in small portions and homogenized before use.

Yeast RNA (Sigma Type VI) was dissolved in H_2O by adding sufficient Tris to maintain the pH at 7.0. The solution was centrifuged at 10,000 rev./min for 10 min, dialyzed at 4°C once against 1% EDTA (pH 7.5) and dialyzed exhaustively against 10 mM Tris-HCl. It was then centrifuged for 4 hours at 226,000 X g_{max} and the supernatant was frozen in small portions. The resulting solution had an A_{260} nm of 785 units/ml and the RNA was of sufficiently low molecular weight that no detectable amount was excluded by Sephadex G-100.

Biochemical Determinations

Amino Acid Incorporation

Incorporation of amino acids into hot trichloroacetic insoluble material was assayed in a 0.5 ml volume at 35°C. Except when noted in the text, each incubation tube contained: 4.48 A_{260} units/ml of polysomes; 0.1 ml of cell sap (diluted as required with Buffer I); 0.05 ml of

a mixture containing 12.5 μM [^{3}H] lysine or [^{3}H] leucine (125 μCi/ml) and 10 μM of each of the other 19 amino acids, 20 mM Tris-HCl (pH 7.4), 150 mM KCl, 0.8 mM ATP, 0.2 mM GTP, 4 mM phosphoenolpyruvate, 1 mM dithioerythritol and 20 μg/ml pyruvate kinase (approximately 10 μM units). All solutions were kept on ice and were added to the incubation tubes in a shaking water bath just prior to timing. Duplicate or triplicate 0.1 ml samples were taken from the reaction mixture at selected time intervals, spotted on 2.3 cm discs of Whatman 3MM paper, placed in cold 10% (w/v) trichloroacetic acid containing 0.2% unlabeled lysine (or leucine) for at least 10 min. The paper discs were washed according to the method originated by Mans and Novelli (1961) and modified by Bollum (1966), including a 5 min wash in hot 5% trichloroacetic acid, and then counted in a liquid scintillation counter in 5 ml of toluene scintillation fluid (4 g/l 2,5-diphenyloxazole and 0.1 g/l 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene) (Bray, 1960). The free amino acids of the cell sap were measured on a Beckman 120C amino acid analyzer. The specific radioactivity of each incubation mixture was corrected according to the free amino acid content of each cell sap sample.

[^{3}H] lysyl-tRNA Incorporation

Cell sap was passed through a DEAE-Sephadex A-25 column prior to assay. Incubations were in 0.5 ml and contained: 4.48 A_{260} units/ml of salt-washed polysomes, 0.1 ml cell sap, 20 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 1 mM dithioerythritol, 150 mM KCl, 0.2 mM GTP and 42,000 dpm [^{3}H] lysyl-tRNA (100 μg tRNA). The reaction mixture was incubated at

35°C. At selected intervals, duplicate 0.1 ml samples were spotted on Whatman 3MM filter paper discs which were then placed in ice-cold 10% trichloroacetic acid containing 0.2% unlabeled lysine, washed and counted as described before.

Aminoacyl-tRNA Synthetase Assay

Aminoacyl-tRNA synthetase activity was assayed by the amino acid-dependent exchange of ^{32}P between ^{32}P labeled pyrophosphate (PPi) and ATP according to the method of Stulberg and Novelli (1962), with some modification. The incubation mixture contained the following components in final volume of 1 ml: 0.1 ml cell sap, 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP, 2 mM ^{32}PPi (10^4-10^5 dpm), 50 mM KF, 1 mM dithioerythritol and 2 mM of each of 20 amino acids. The reaction was initiated by the addition of cell sap and incubated at 35°C for 15 min. The reaction was terminated by the addition of 0.5 ml of 7% $HClO_4$ followed by 0.2 ml of a suspension of Norit A (200 mg dry weight). Four milliliters of 0.1 M sodium acetate were added to the mixture which was then mixed thoroughly with a wooden applicator stick. The adenosine phosphates were absorbed by the Norit A and inorganic phosphates were left in the solution. Norit A was separated by low-speed centrifugation, resuspended in 5 ml of 0.5 M acetate buffer plus 0.1 M PPi (pH 4.5), mixed thoroughly, centrifuged and the supernatant discarded. The washing procedure was repeated 3 times with 5 ml of 0.05 M acetate, pH 4.5 (no PPi) and once with 5 ml of H_2O. The Norit was then suspended in 5.0 ml of 1N HCl and heated at 100°C for 15 min to hydrolyze the terminal phosphate groups of ATP. After hydrolysis, the mixture was centrifuged

and 1 ml aliquots of the supernatant were removed and then counted in a liquid scintillation counter.

Ribonuclease Assay

The ribonuclease assay is essentially a modification of the procedure of Anfinsen *et al.* (1954), a method based on the liberation of acid-soluble oligonucleotides from yeast RNA. The RNA substrate was purified by the method of Utsunomiya and Roth (1966). Yeast RNA (Sigma Type VI) was dissolved in H_2O and the pH was adjusted to 7.0 by adding Tris. The solution was dialyzed against 5 changes of 2.5 volumes of 0.025 M EDTA (pH 7.0) and then against 5 changes of 5.0 volumes of H_2O. The concentration of the dialyzed RNA was adjusted to 1% as determined by absorbance measurement. The purified RNA thus obtained was stored frozen until use and this material gave very low blank value. Ribonuclease activity was measured by incubating, at 35°C for 1 hour, 0.4 ml of 1% purified yeast RNA, 0.1 ml of 200 mM Tris-HCl (pH 7.4) and 0.5 ml of enzyme preparation. Triplicate samples were assayed when possible. The reaction was terminated by the addition of 0.5 ml ice-cold 0.75% uranyl acetate in 25% $HClO_4$. The mixture was kept at 0°C for at least 15 min. After centrifugation at low speed, the supernatant was diluted 10 times with H_2O and the absorbance at 260 nm was measured in a Beckman DU spectrophotometer against a zero-time blank (containing both enzyme sample and RNA). Another blank without enzyme was treated in the same way in order to correct for non-enzymatic depolymerization of RNA.

Ribonuclease Inhibitor Assay

Ribonuclease inhibitor activity was measured according to the method of Roth (1956) with some modification. A final volume of 1.0 ml contained: 0.1 ml of a solution containing 0.0125 µg of pancreatic ribonuclease, 0.4 ml of 1% purified yeast RNA, 0.1 ml of 200 mM Tris-HCl (pH 7.4) and 0.4 ml of cell sap. The mixture was incubated at 35°C for 60 min and the reaction was terminated by the addition of 0.5 ml of 0.75% uranyl acetate in 25% $HClO_4$. The A_{260} of the sample was measured as described previously. Controls containing 0.4 ml of Buffer I instead of cell sap, and others containing 0.1 ml of H_2O in place of pancreatic ribonuclease were assayed simultaneously.

Analysis of Cell-Free Reaction Products

For analysis of protein products, a 2-5 ml reaction mixture was used. Each ml of reaction mixture contained 5-10 A_{260} units of membrane-bound or free polysomes, 1 mg cell sap protein, 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 6 mM $MgCl_2$, 0.8 mM ATP, 0.2 mM GTP, 4 mM phosphoenolpyruvate, 20 µg pyruvate kinase, 1 mM dithioerythritol, 1 µM each of [^{3}H] lysine and [^{3}H] leucine (12.5 µCi per ml reaction mixture), 1 µM of each of the other 18 amino acids. Incubation was performed at 35°C for 60 min. Subsequent treatment of the sample was carried out essentially by the method of Koga and Tamaoki (1974) At the end of the incubation, the reaction mixture was centrifuged at 150,000 X g_{max} for 1.5 hours to sediment polysomes. The supernatant was dialyzed extensively against several changes of 20 mM Tris-HCl (pH 7.4) and then subjected to poly-

acrylamide gel electrophoresis. The pellet was resuspended in the original volume of 20 mM Tris-HCl (pH 7.4). The sample was frozen in dry ice-acetone, thawed and sonicated for 5 min. This treatment was repeated three times. The disrupted sample was centrifuged at 150,000 X g_{max} for 1.5 hours and the resulting supernatant was dialyzed as above and subjected to gel electrophoresis. Duplicate 10 µl samples were taken from the reaction mixture in each step and spotted on filter paper discs. Hot trichloroacetic acid insoluble materials were determined as previously described.

In case of the 3,000 X g supernatant system, incubation for amino acid incorporation was the same as with the polysomal system except that 0.2 g equivalent of liver per ml reaction mixture was substituted for polysomes and cell sap. Subsequent analysis of the reaction products was as described above.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis in the absence of SDS was performed in 7.5% gels (acrylamide:bisacrylamide, 37.5:1) at pH 8.9 according to the method of Davis (1964). Between 5,000 to 15,000 cpm of hot trichloroacetic acid insoluble material was applied to the gels. The current was adjusted to 2 m Amp per tube for the first 20 min, then it was increased to 5 m Amp per tube. Total running time was 2 hours. After electrophoresis, the gels were sliced into 2 mm sections which were dissolved by incubating in 0.2 ml of 30% H_2O_2 at 60°C in tightly capped scintillation vials and then counted in 10 ml of Aquasol scintillation fluid.

SDS-polyacrylamide gel electrophoresis was performed in 7.5% gel at pH 7.1 according to the method of Palmiter *et al.* (1971). The current

was adjusted to 2 m Amp per tube for the first 20 min, then it was increased to 5 m Amp per tube. Total running time was 4 hours. Radioactivity in the gel was determined as described above.

Immunoprecipitation

A monospecific antiserum to chicken serum albumin was produced in a goat by three alternate weekly intramuscular injections of purified chicken albumin. Fifty microliters of the antiserum would precipitate between 6 to 12 µg of albumin at the equivalence point. For immunoprecipitation of the cell-free reaction products, 10 µg of purified albumin were mixed with 100-200 µl of sample followed by the addition of 100 µl of antialbumin serum. The mixture was incubated at 35°C for 60 min and then at 4°C for 24 hours. The sample was centrifuged at low speed for 10 min at 4°C. The precipitate was washed three times with 20 mM Tris-HCl (pH 7.4) in 150 mM NaCl, and dissolved in 1% SDS, 1% β-mercaptoethanol and 20 mM Tris-HCl (pH 7.4) by boiling for 5 min. Sucrose to a final concentration of 10% and Bromphenol blue tracking dye were added to the sample and SDS gel electrophoresis was performed as described above.

For assay of albumin synthesis, immunoprecipitation was performed as described above except in the presence of 1% sodium deoxycholate and 1% Triton X-100 to reduce nonspecific precipitation (Shafritz, 1974b). The immunoprecipitate was washed three times with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate and 1% Triton X-100 and then dissolved in 0.04 N NaOH. The radioactivity of this solution was determined by counting in 10 ml of Aquasol scintillation fluid. A blank containing

nonimmune goat serum was treated under the same conditions and its radioactivity subtracted from the radioactivity in the immunoprecipitate.

Sucrose Gradient Centrifugation

Linear sucrose gradients (36 ml, 15-40% sucrose in Buffer I) were prepared in an ISCO, Model 570, gradient former. Approximately 2.0 to 5.0 A_{260} units of polysomes were applied to each gradient and centrifuged at 131,000 X g_{max} for 2 hours in a Spinco SW 27 swinging bucket rotor. The bottom of each tube was punctured and the gradient was displaced upwards with a 50% sucrose solution. The gradients were monitored at 254 nm with an ISCO Model UA-4 ultraviolet analyzer.

Measurement of RNA and Protein

RNA concentration was determined by the method of Fleck and Munro (1962). Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical Analyses

The results were expressed as mean value $\pm$ the standard error when more than three separate experiments were done. Other results were expressed as the average of duplicate or triplicate determinations when the values were derived from a single representative experiment.

RESULTS AND DISCUSSION

Characteristics of the Cell-Free Protein-Synthesizing System Derived from Embryo Livers at Various Stages of Development

Requirements of Cell-Free Amino Acid Incorporation

The cell-free amino acid incorporating system used in this investigation was composed of polysomes and cell sap prepared from chick embryo livers. Various conditions were tested to achieve maximal activity, and the method of preparation outlined in Materials and Methods was found to be most satisfactory. KCl concentrations of 150-250 mM in the isolation medium resulted in higher polysome yield and activity than 25 mM. In this study, 150 mM KCl rather than 250 mM KCl was used because higher KCl concentration might increase the risk of ribosome dissociation. Preliminary experiments showed polysomes isolated in 150 mM KCl to be slightly more active than those isolated in 250 mM KCl. $MgCl_2$ concentrations below 5 mM in the isolation buffer reduced the yield of polysomes. The use of more concentrated sucrose (2.0 M versus 1.5 M) in the lower layer of the discontinuous gradients eliminated most of the protein impurities. Polysomes isolated from livers of embryos older than 11 days showed degradation by nuclease. Addition of bentonite and low molecular weight yeast RNA to the isolation buffer as nuclease inhibitors protected polysomes from degradation. Bentonite or yeast RNA alone

reduced the nuclease activity; however, a combination of the two proved best.

Incorporation of amino acid into hot trichloacetic acid insoluble material depended on the presence of polysomes, cell sap and an energy source (Table 1). Figure 4 shows the effect of increasing amounts of cell sap on amino acid incorporation by a fixed amount of polysomes. Figure 5 represents another type of experiment in which the amount of cell sap was fixed and increasing amounts of polysomes were added. Within the concentration ranges tested, incorporation was linearly dependent upon the amount of both cell sap and polysomes. The system could not be saturated with cell sap probably because one or more active components were in low concentration. Use of lower polysome levels would have reduced the lysine incorporation to less than significant levels. A typical time course of incorporation is shown in Figure 6. The incorporation was linear with time for about 10 minutes.

Polysome Activity

The amino acid incorporating activity of cell-free systems derived from embryos of different ages was compared. Polysomes prepared from 12-day and 19-day embryonic livers were incubated with homologous cell sap. As shown in Figure 7, the cell-free system derived from 12-day embryos was found to be more active than that from 19-day embryos. To investigate this difference further, the activity of polysomes derived from embryos of various ages was compared. For this purpose, the assays were set up so that the polysome age was the

Table 1

Dependence of Protein Synthesis on Various Components of the Reaction Mixture

Polysomes and cell sap were prepared from 17-day embryos and incubated for 15 min at 35°C in the complete reaction mixture described in Materials and Methods with 0.1 mg cell sap protein. Incorporation of [^{3}H] lysine was determined as acid soluble material assuming 380 cpm = 1 pmol lysine incorporated. The values given are the means of triplicate reaction mixtures assayed in triplicate.

System	pmoles of [^{3}H] Lys incorpd/mg rRNA
Complete	364.0
Complete less polysomes	10.6
Complete less cell sap	2.8
Complete less energy source*	3.4

*ATP, GTP, Phosphoenolpyruvate

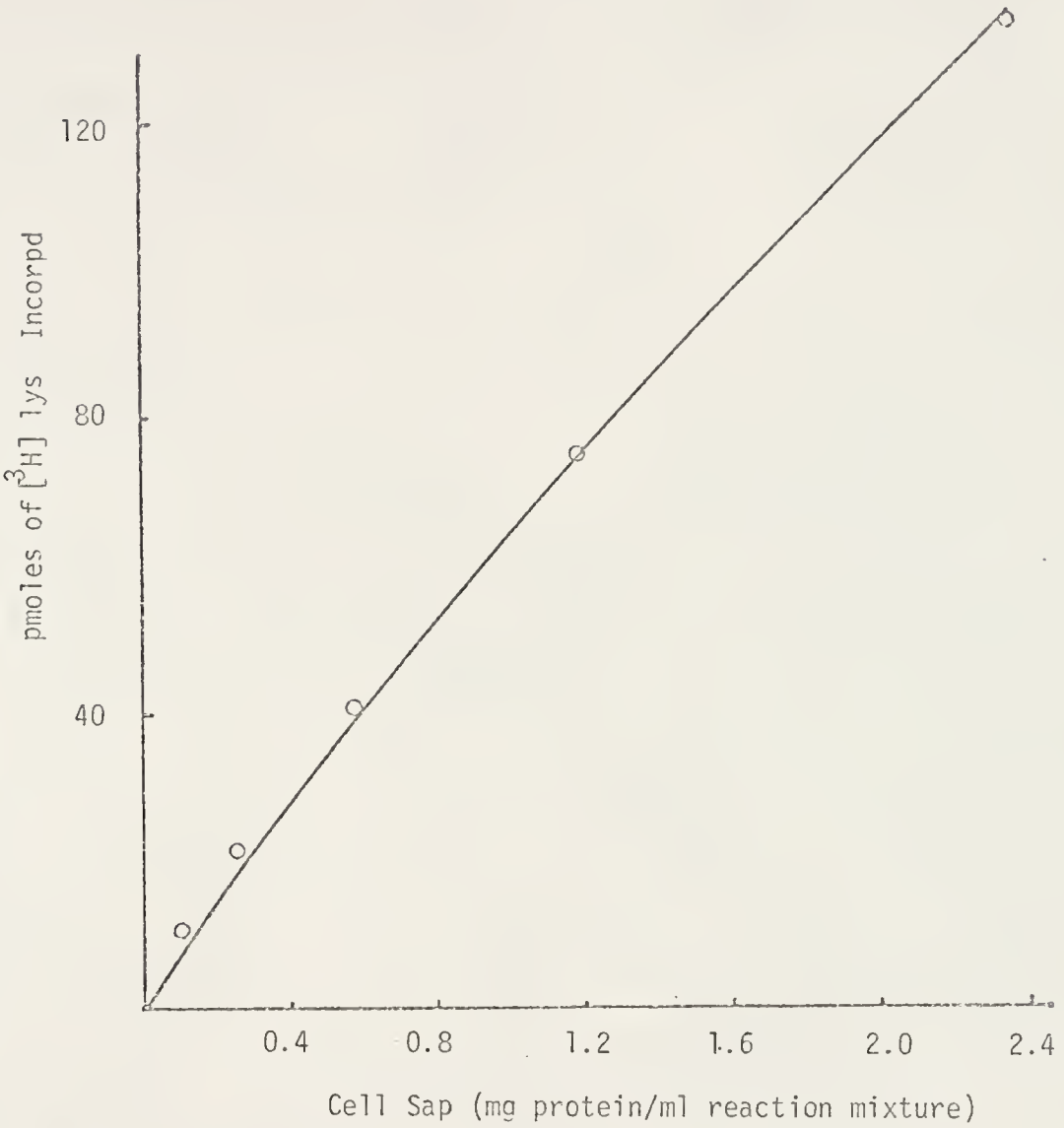


Figure 4. Effect of cell sap concentration on the [^{3}H] lysine incorporation into protein. Polysomes and cell sap were prepared from 19-day embryos. Incubation was performed at 35°C for 15 min as described in Materials and Methods with 0.2 mg ribosomal RNA and indicated level of cell sap protein per ml reaction mixture. Each point represents the average of duplicate reaction mixtures assayed in triplicate.

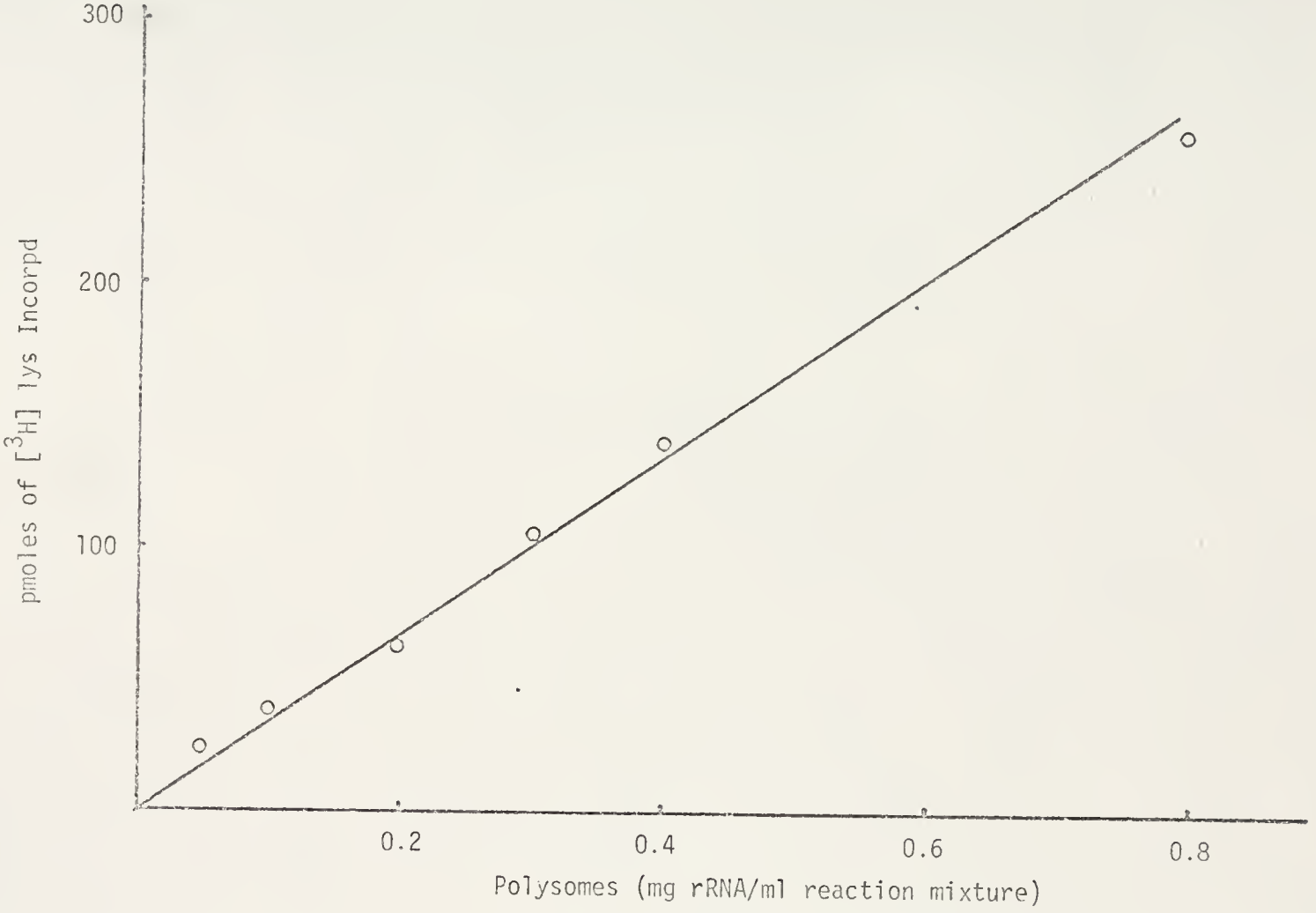


Figure 5. Effect of polysome concentration on the [^{3}H] lysine incorporation into protein. Polysomes and cell sap were prepared from 19-day embryos. Incubation was carried out at 35°C for 15 min as described in Materials and Methods with 1.0 mg cell sap protein and indicated level of ribosomal RNA per ml reaction mixture. Each point represents the average of duplicate reaction mixtures assayed in triplicate.

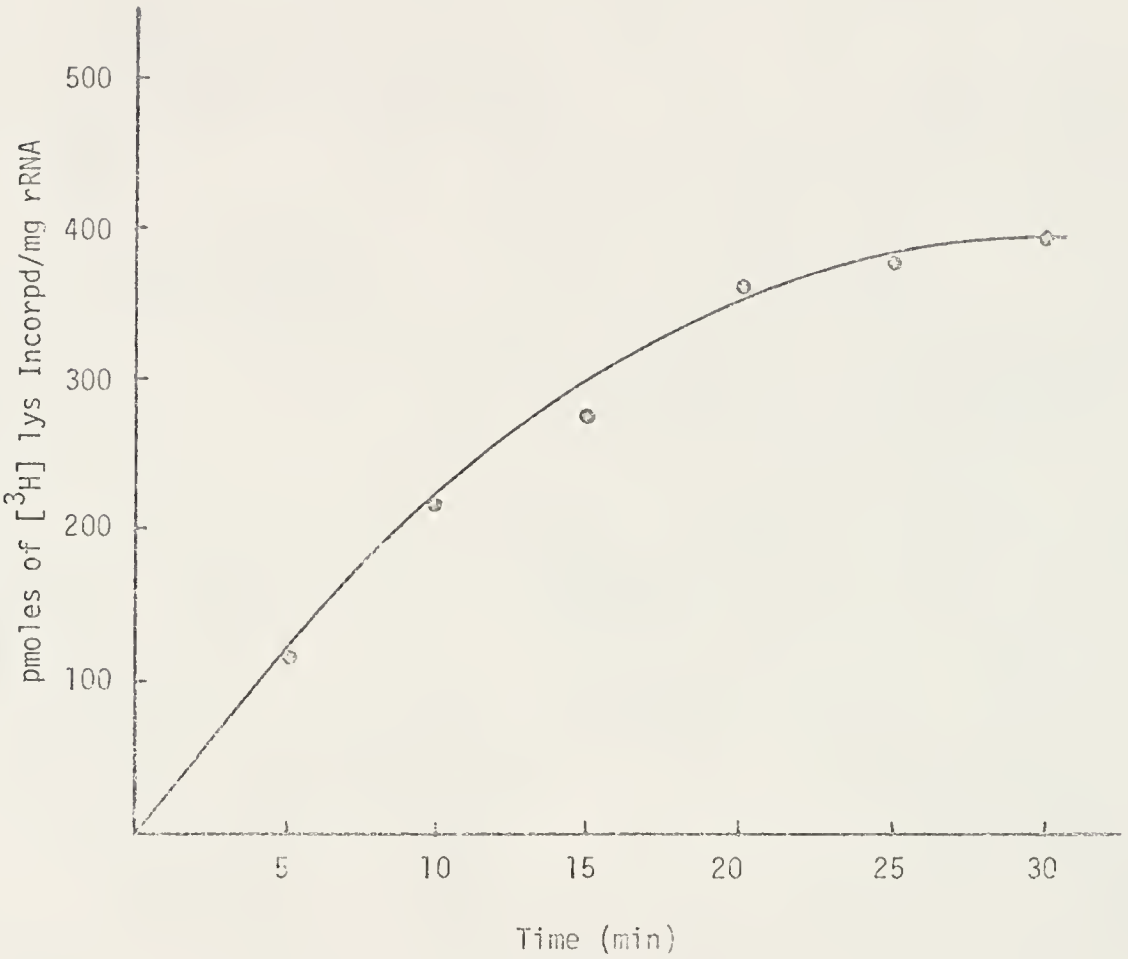


Figure 6. Time course of [^{3}H] lysine incorporation into protein. Polysomes and cell sap were prepared from 17-day embryos. Incubation was performed as described in Materials and Methods except that 2.0 ml total volume was used. Each ml reaction mixture contained 1.0 mg cell sap protein and 0.2 mg ribosomal RNA. The values given are the means of duplicate reaction mixtures assayed in duplicate at each time interval. Zero-time blanks have been subtracted from each point.

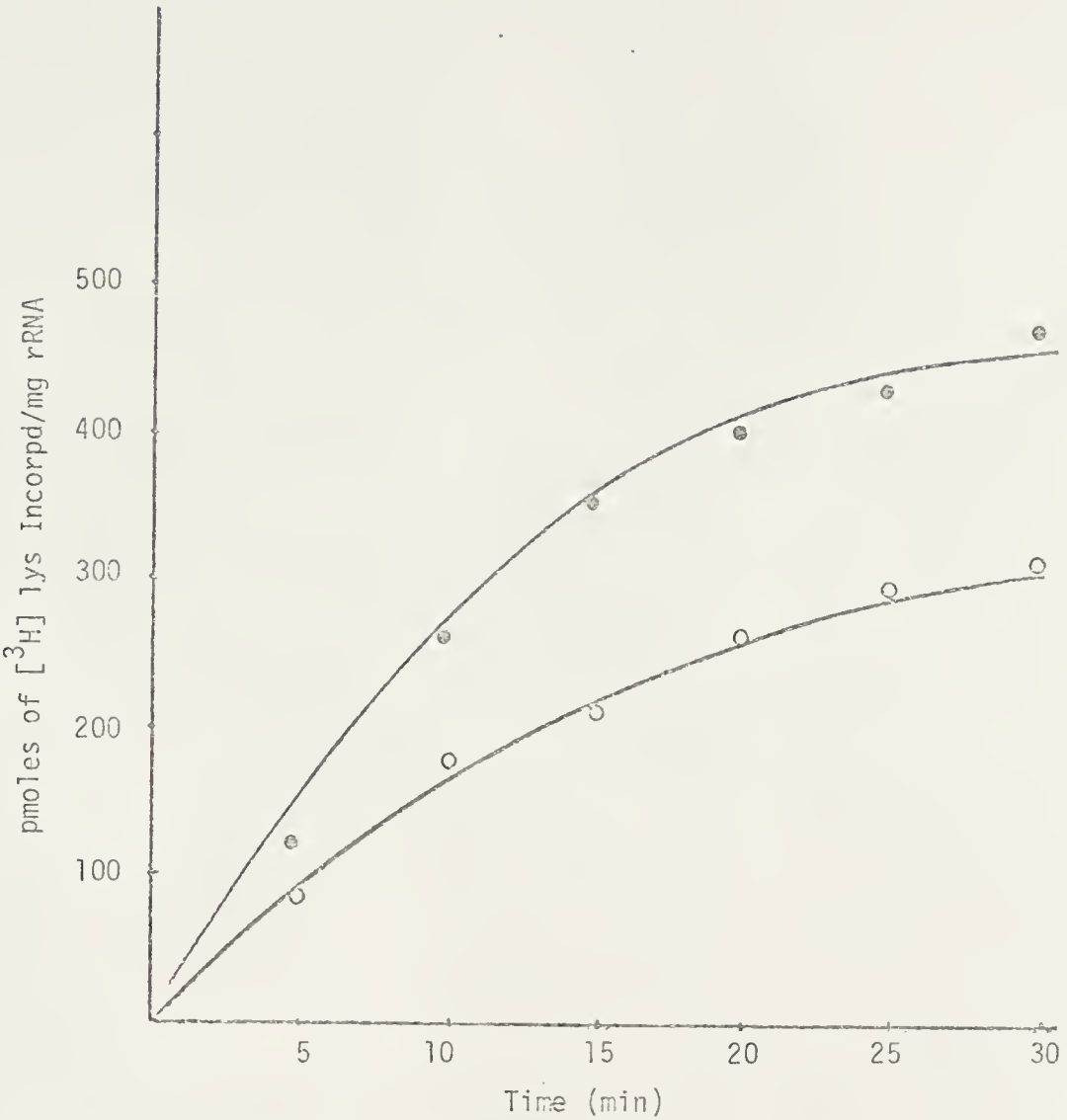


Figure 7. Amino acid incorporating activity of cell-free systems derived from 12-day and 19-day embryos. Polysomes prepared from 12-day or 19-day embryonic livers were incubated with homologous cell sap (1.0 mg cell sap protein per ml reaction mixture) as described in Materials and Methods. The incubation was for various times at 35°C. The values given are the means of triplicate reaction mixtures assayed in duplicate. ●, 12-day embryos; ○, 19-day embryos.

only variable in the reaction mixture. Polysomes from embryos of different ages were prepared at the same time and incubated in the presence of a standard cell sap preparation. As can be seen in Figure 8, no significant differences in polysome activity were observed. The sucrose gradient profiles of these polysome preparations are shown in Figure 9. High molecular weight polysomes can be isolated from embryonic livers throughout the developmental period studied. The ratio of polysomes (10-30 ml from meniscus) to monomers (6 ml from meniscus), indicated by the area under the profiles, decreased with increasing developmental age. The polysome peak was found to be nearer the meniscus in sucrose density gradients with increasing age, indicating a decrease in average polysome length with maturity. Since these polysomes were isolated, resuspended and then analyzed by sucrose gradients, subtle differences in the profiles may be due to minor variations in the complex isolation procedure.

Cell Sap Activity

The activity of cell saps derived from embryos of various ages was also compared using a standard polysome preparation. The concentration of free amino acid in each cell sap preparation varied quite significantly which caused significant changes in the specific radioactivity of each reaction mixture. Therefore the free amino acid concentrations of each cell sap sample were determined in a Beckman amino acid analyzer and used for corrections in the specific radioactivities when calculating activities of different cell sap samples. When such corrections were made, the incorporation of amino acid was

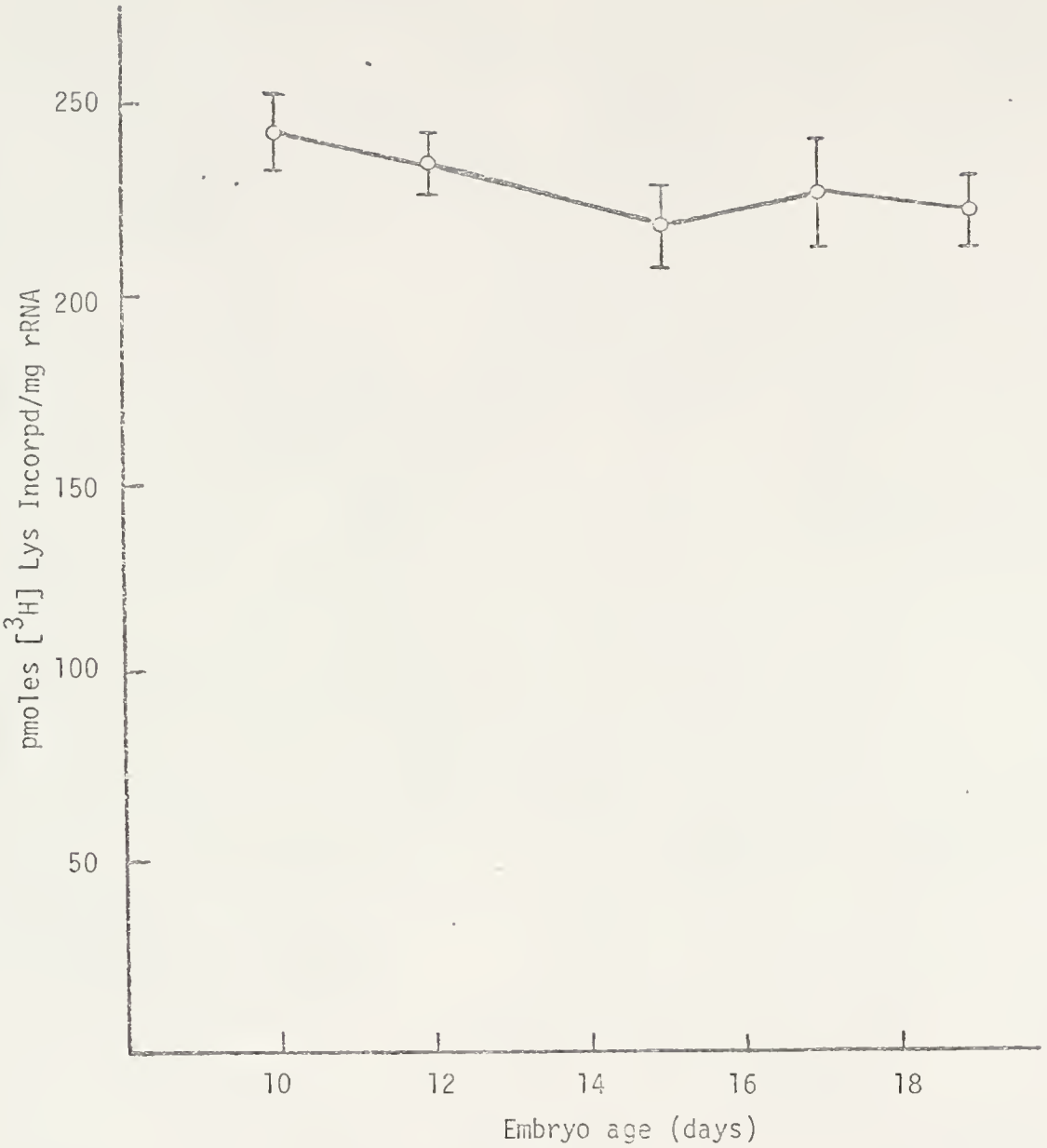
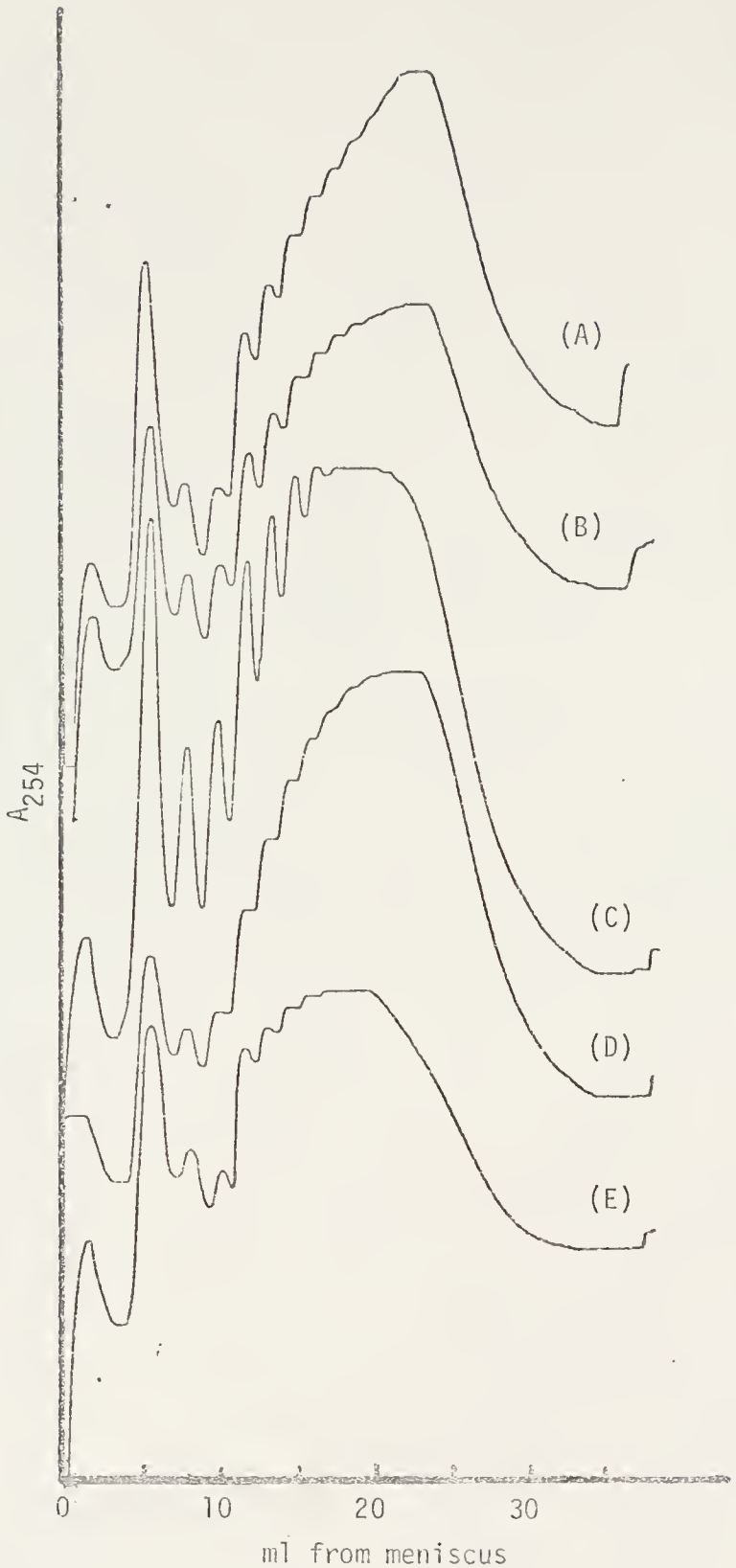


Figure 8. Amino acid incorporation by total polysomes derived from embryos at various stages of development. Polysomes were isolated from five different age embryos on the same day. Each polysome preparation was incubated with cell sap prepared from 19-day embryos (1.0 mg cell sap protein/ml reaction mixture) and other components as described in Materials and Methods. Incubation was for 15 min at 35°C. The values given are the means ± S.E. of three samples of each age.

Figure 9. Sucrose gradient profiles of total polysomes derived from various age embryos. Polysomes were prepared as described in Materials and Methods. Between 2 to 4 A_{260} units of polysomes were layered on each 15-40% sucrose gradient. The gradients were centrifuged and monitored as described in Materials and Methods. (A) 10-day polysomes, (B) 12-day polysomes, (C) 15-day polysomes, (D) 17-day polysomes, (E) 19-day polysomes.

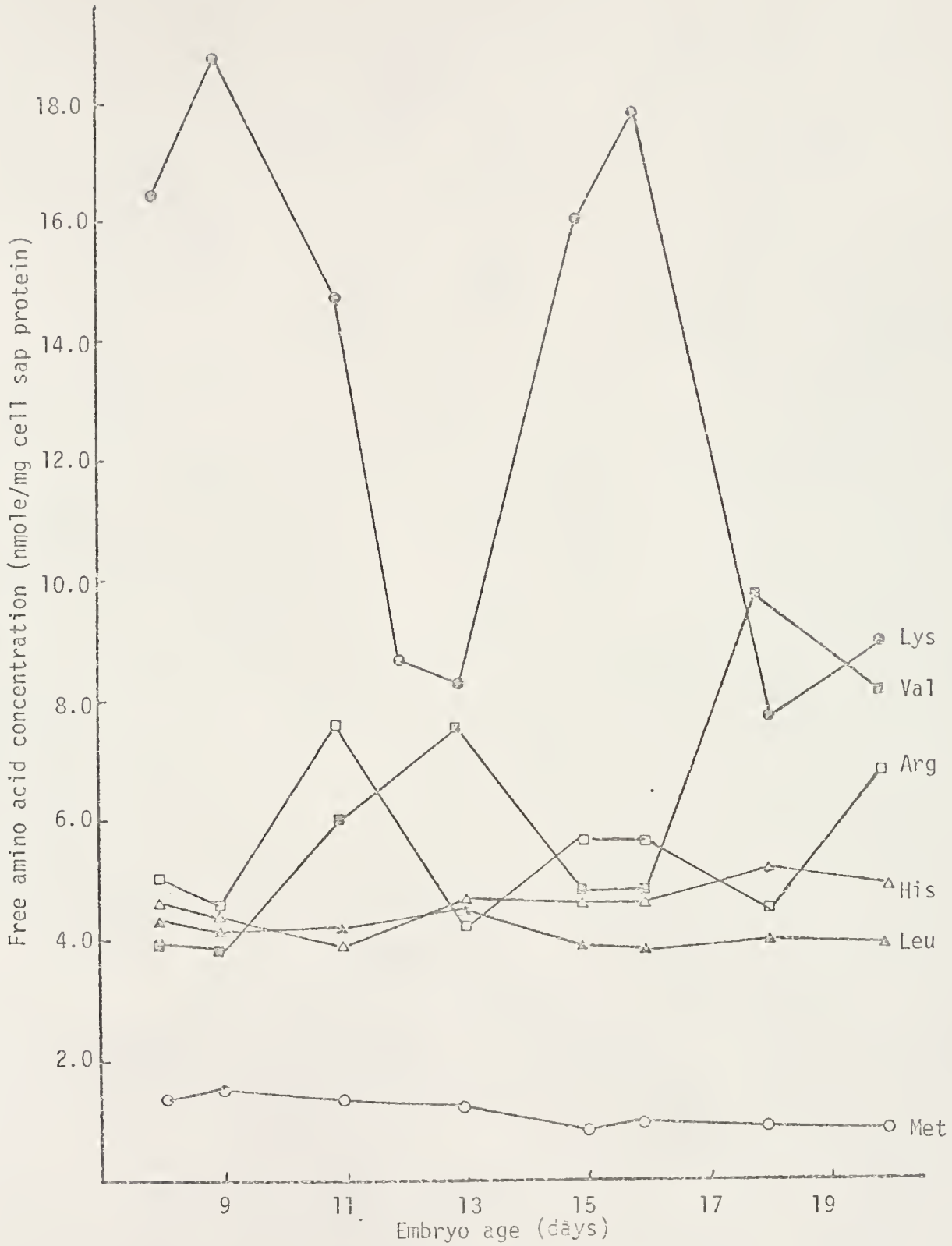

A254
(A)
(B)
(C)
(D)
(E)
0
10
20
30
ml from meniscus

proportional to cell sap concentration in the range usually assayed. In all assays of cell sap activity, duplicate determinations at three different cell sap concentrations were done to assure that comparisons were made at the linear part of the incorporation versus cell sap concentration curve.

At the beginning of this study, [^{3}H] lysine was used for all incorporation studies. During the analyses of cell sap samples, two interesting observations were made. First, the free lysine concentration in the cell sap appeared to be age-dependent. Lysine concentrations of 12-13 day and 18-20 day cell sap were always considerably lower than the other days (Figure 10). In order to investigate whether the concentrations of other amino acids were also age-dependent, complete amino acid analyses were performed for all cell sap samples. Some representative results are shown in Figure 10. The concentrations of leucine, histidine and methionine was constant throughout development, while those of other amino acids showed variations with age. The patterns of arginine and lysine were similar in that the variations in concentration were parallel.

The second observation was that the lysine peak from embryonic cell sap, as eluted from the amino acid analyzer, was not symmetrical. As can be seen in Figure 11, it appeared that another component of cell sap eluted a little earlier than lysine did and formed a shoulder on the lysine peak. Attempts to identify this other component by comparing with several possible known compounds were unsuccessful. Changing the pH of the eluting buffer did not further separate the two components. It is interesting that the phenomenon is not observed

Figure 10. Free amino acid content of the cell sap derived from embryos at various stages of development. Cell sap proteins were precipitated with 10% trichloroacetic acid and the resulting supernatant was analyzed on a Beckman 120C amino acid analyzer. The values represent the average of at least three determinations. The lysine values were the average of 5 to 7 determinations. ●, lysine; ■, valine; □, arginine; △, histidine; ▲, leucine; ○, methionine.


Free amino acid concentration (nmole/mg cell sap protein)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
9
11
13
15
17
19
Embryo age (days)
Lys
Val
Arg
His
Leu
Met

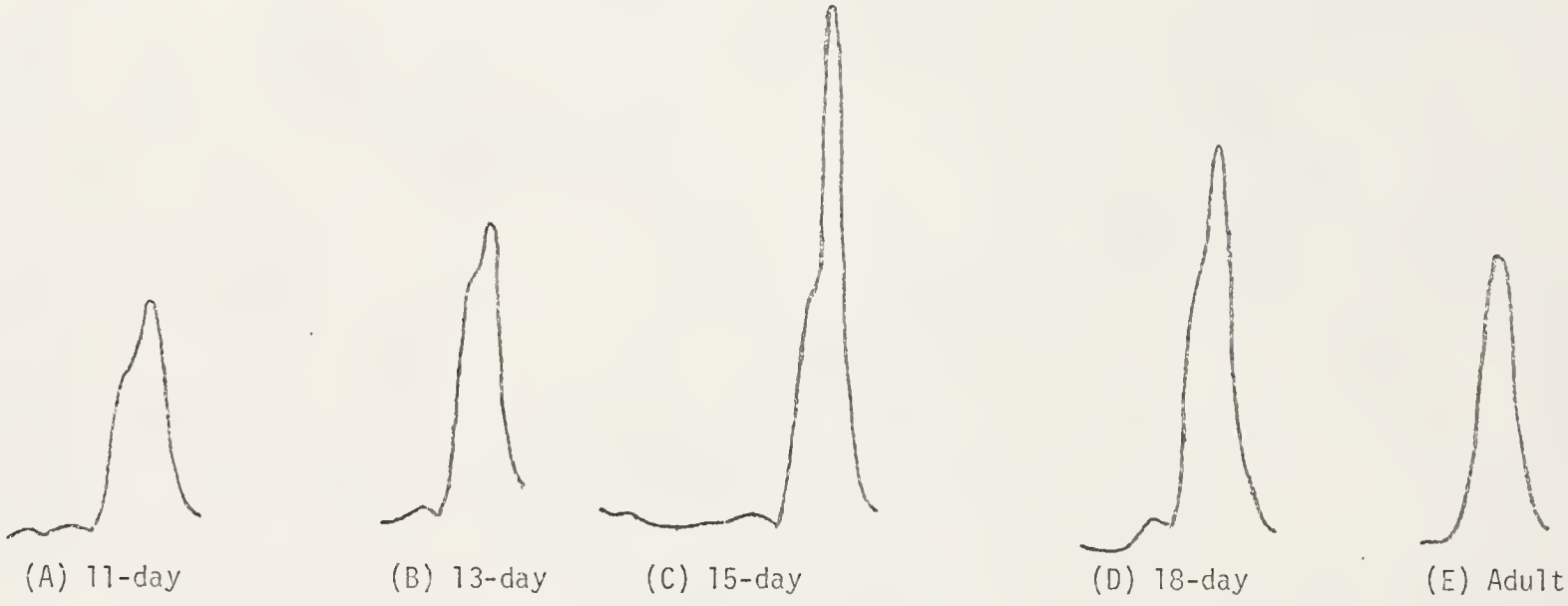


Figure 11. Lysine profiles from amino acid analysis. (A) 11-day cell sap, (B) 13-day cell sap, (C) 15-day cell sap, (D) 18-day cell sap, (E) adult cell sap.

in cell sap from adult chicken liver. At this point, all that can be said is that the cell sap from embryonic chick liver apparently contains a ninhydrin-positive component which is either absent or present in very low concentration in the cell sap from adult chicken liver.

To ascertain that the measurement of lysine concentration did not introduce artifacts in the assay of cell sap activity, [^{3}H] leucine was used in some experiments. Figure 12 shows the cell sap activities at different stages of development using either [^{3}H] lysine or [^{3}H] leucine. Cell saps from young embryos were more active in amino acid incorporation than those from older embryos. Similar results were obtained with either labeled amino acid. This indicates that correction of the specific radioactivity by amino acid analysis is reliable. Figure 13 shows the effect of cell sap concentration on the incorporating activity of cell sap prepared from two different age embryos. The age-related activity difference was observed at all concentrations tested.

It is known that supernatant factors tend to bind to crude polysomes and that they can be removed by washing polysomes with 0.5 M KCl. The factors bound to crude polysomes may introduce a significant background incorporation when cell sap concentration is limiting and this background incorporation may interfere with cell sap activity measurements. To test this possibility, cell sap activity was studied using 0.5 M KCl-washed polysomes. The results are shown in Figure 12. Incorporation was reduced considerably as compared with that measured using crude polysomes, but similar age-related changes were obtained.

The above experiments suggest that cell sap from young embryos is more active in amino acid incorporation than that from embryos at

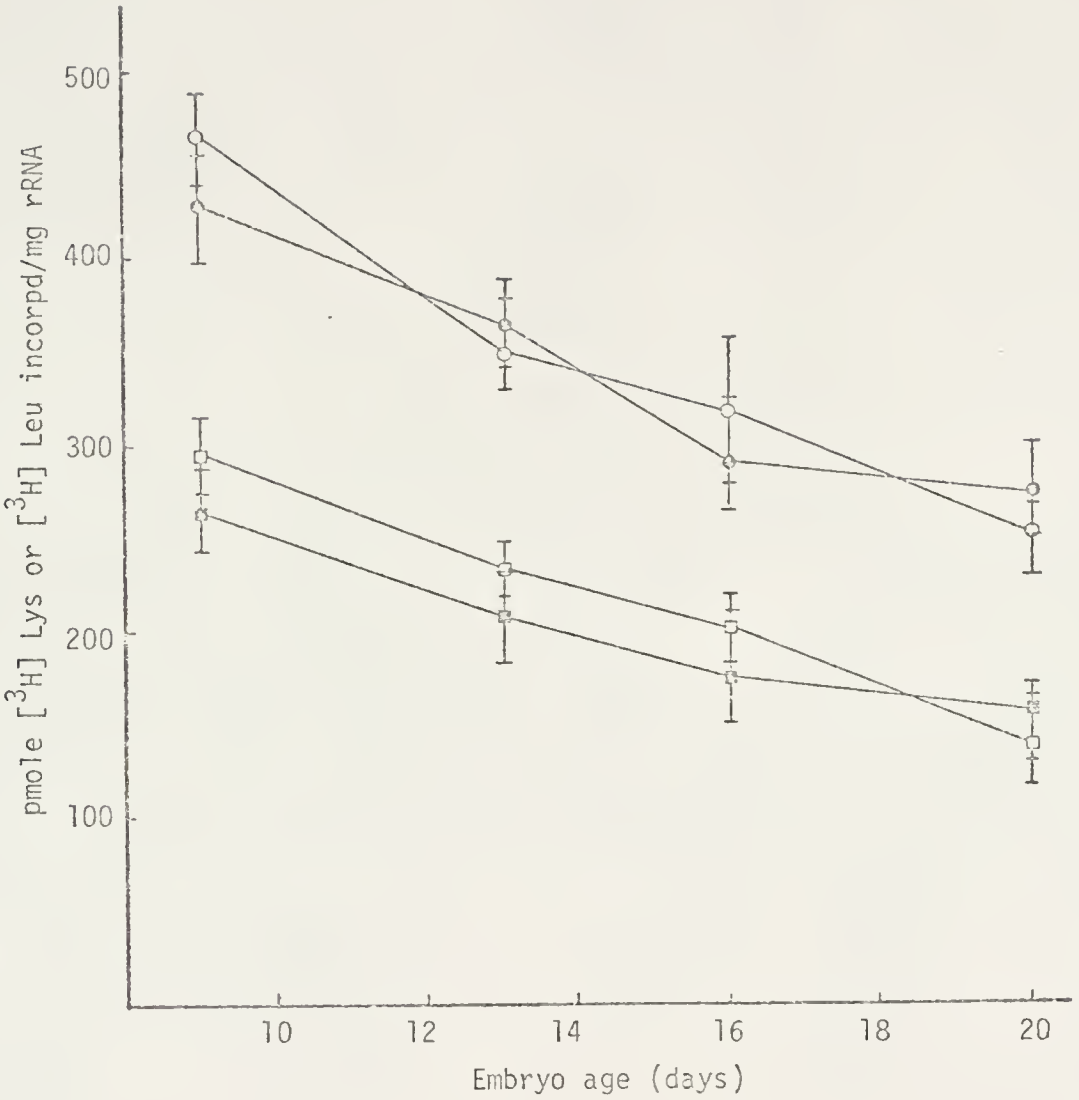


Figure 12. Amino acid incorporating activity of cell saps derived from embryos of various ages. Cell saps prepared from livers of different age embryos were incubated with a standard crude or salt-washed polysome preparation at 35°C for 15 min as described in Materials and Methods. The activity was determined using three different cell sap concentrations (between 0.5 and 1.5 mg protein per ml reaction mixture). The results have been normalized to incorporation per mg cell sap protein. Each point represents the mean + S.E. of three separate experiments. The activity was measured with either [^{3}H] lysine or [^{3}H] leucine. ●, [^{3}H] lysine incorporation, crude polysomes; o, [^{3}H] leucine incorporation, crude polysomes; ■, [^{3}H] lysine incorporation, salt-washed polysomes; □, [^{3}H] leucine incorporation, salt-washed polysomes.

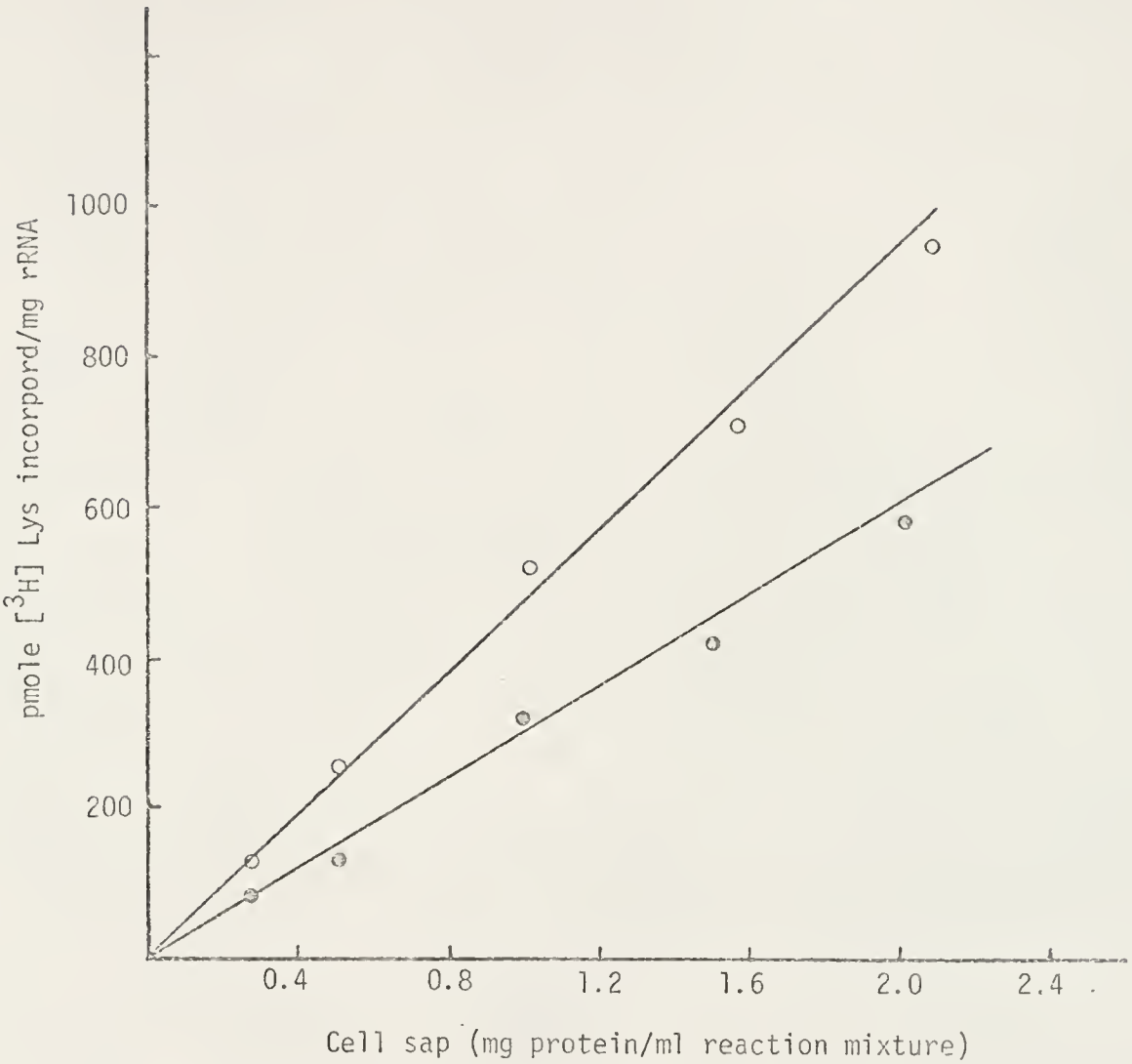


Figure 13. Cell sap dependence of [^{3}H] lysine incorporation. Polysomes prepared from 16-day embryonic livers were incubated with cell sap prepared from 9-day or 16-day embryonic livers under the conditions described in Materials and Methods. Each point represents the average of duplicate reaction mixtures assayed in triplicate. o, 9-day cell sap; ●, 16-day cell sap.

later stages of development. The results of Duck-Chong *et al.* (1964) also showed that liver slices from young embryos incorporated amino acid more rapidly than those from old embryos. Whether the decline in amino acid incorporation reflects the *in vivo* protein synthesizing capacity is not clear. One possible correlation seems to be with the decrease in growth rate of liver during development (Romanoff, 1967, p. 267).

[^{3}H] lysyl-tRNA Incorporation

Cell sap contains enzymes and other factors which are required for different steps in protein synthesis. In the following experiment, the ability of cell sap to transfer labeled amino acid from aminoacyl-tRNA into polypeptide was tested. [^{3}H] lysyl-tRNA was prepared by charging chick liver tRNA with [^{3}H] lysine. To avoid differential dilution of added [^{3}H] lysyl-tRNA with endogenous lysyl-tRNA, the cell sap was passed through a DEAE-Sephadex A-25 column to remove the endogeneous tRNAs. The extent of incorporation was assayed using a salt-washed polysome preparation. Under these conditions, the incorporation of radioactivity was linear with time for at least 15 minutes and was dependent upon the amount of cell sap added (Figures 14 and 15).

The incorporation by cell sap derived from embryos of different ages is shown in Figure 16. Cell sap from young embryos was more active in the transfer of amino acid into polypeptide than cell sap from older embryos. This is consistent with the previous observation when labeled amino acids were used for incorporation, although the age-related differences were not exactly parallel.

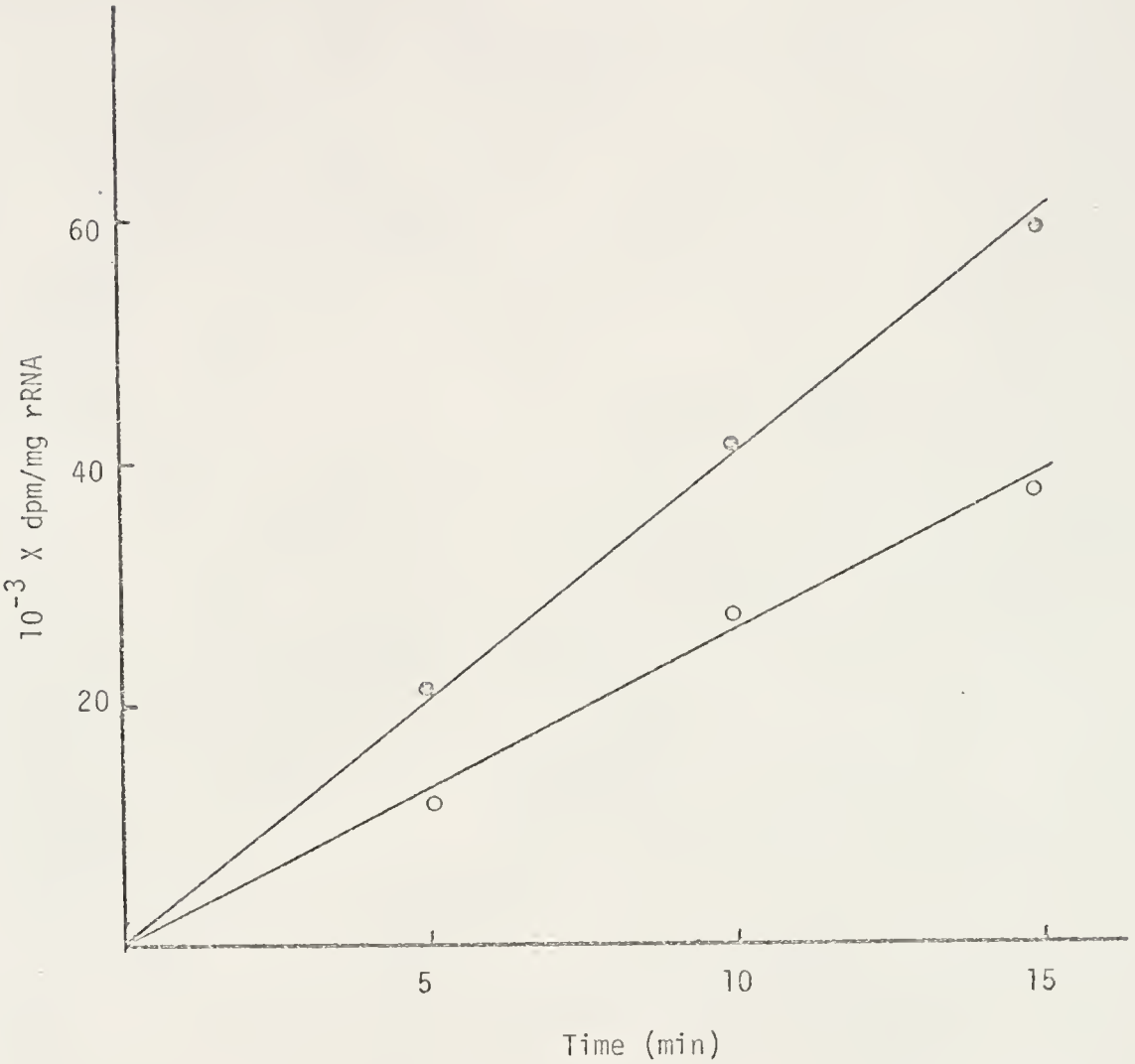


Figure 14. Time course of incorporation of [^{3}H] lysine from [^{3}H] lysyl-tRNA into polypeptide. Salt-washed polysomes prepared from 20-day embryonic livers were incubated with the cell sap prepared from 13- or 20-day embryonic livers under the conditions described in Materials and Methods. Each ml reaction mixture contained 1.0 mg cell sap protein. The values given are the means of triplicate determinations. ●, 13-day cell sap; o, 20-day cell sap.

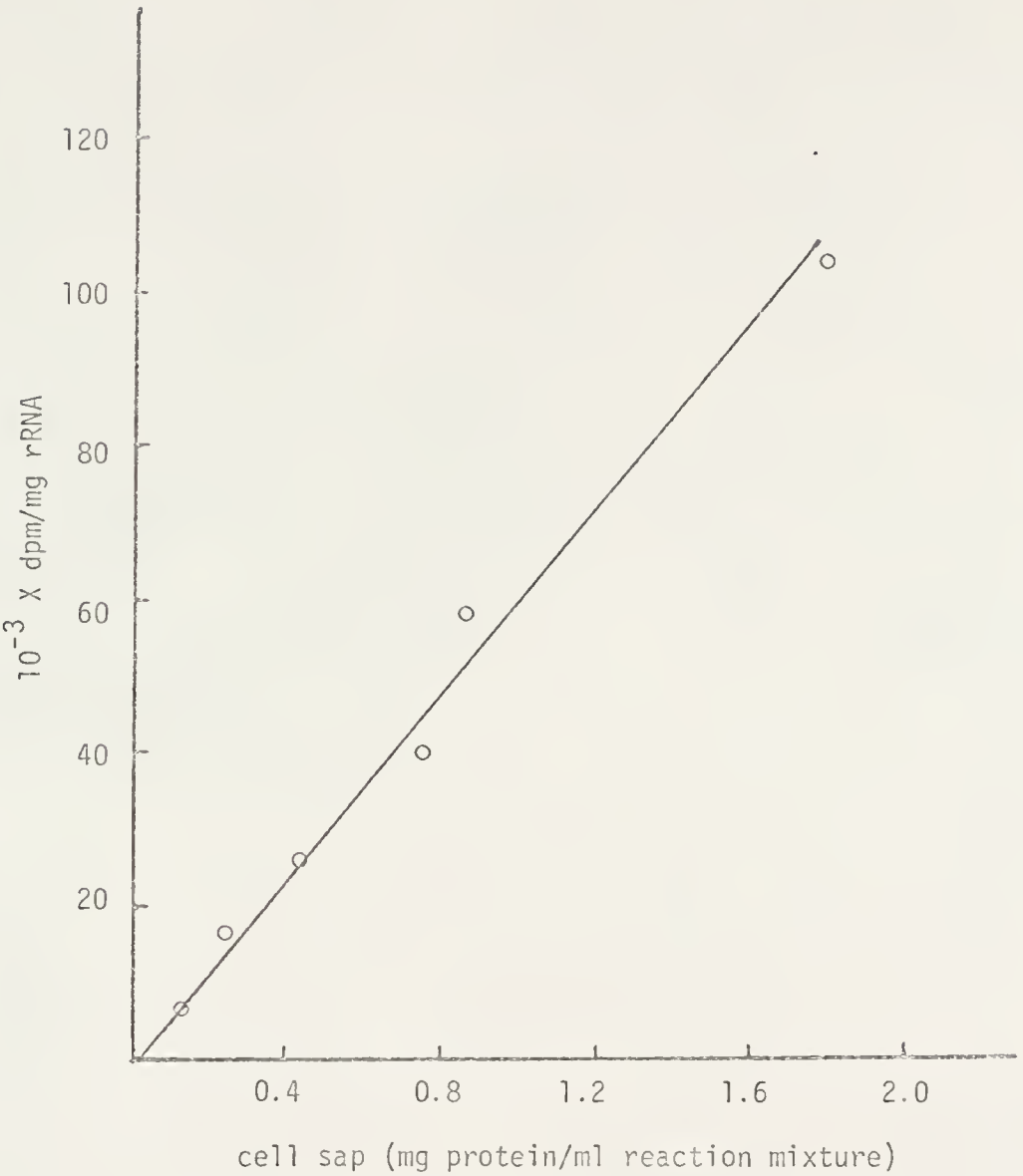


Figure 15. Effect of cell sap concentration on the incorporation of [^{3}H] lysyl-tRNA into polypeptide. Polysomes and cell sap were prepared from 16-day embryonic livers. The reaction conditions have been described in Materials and Methods. Each point represents the average of duplicate reaction mixtures assayed in triplicate.

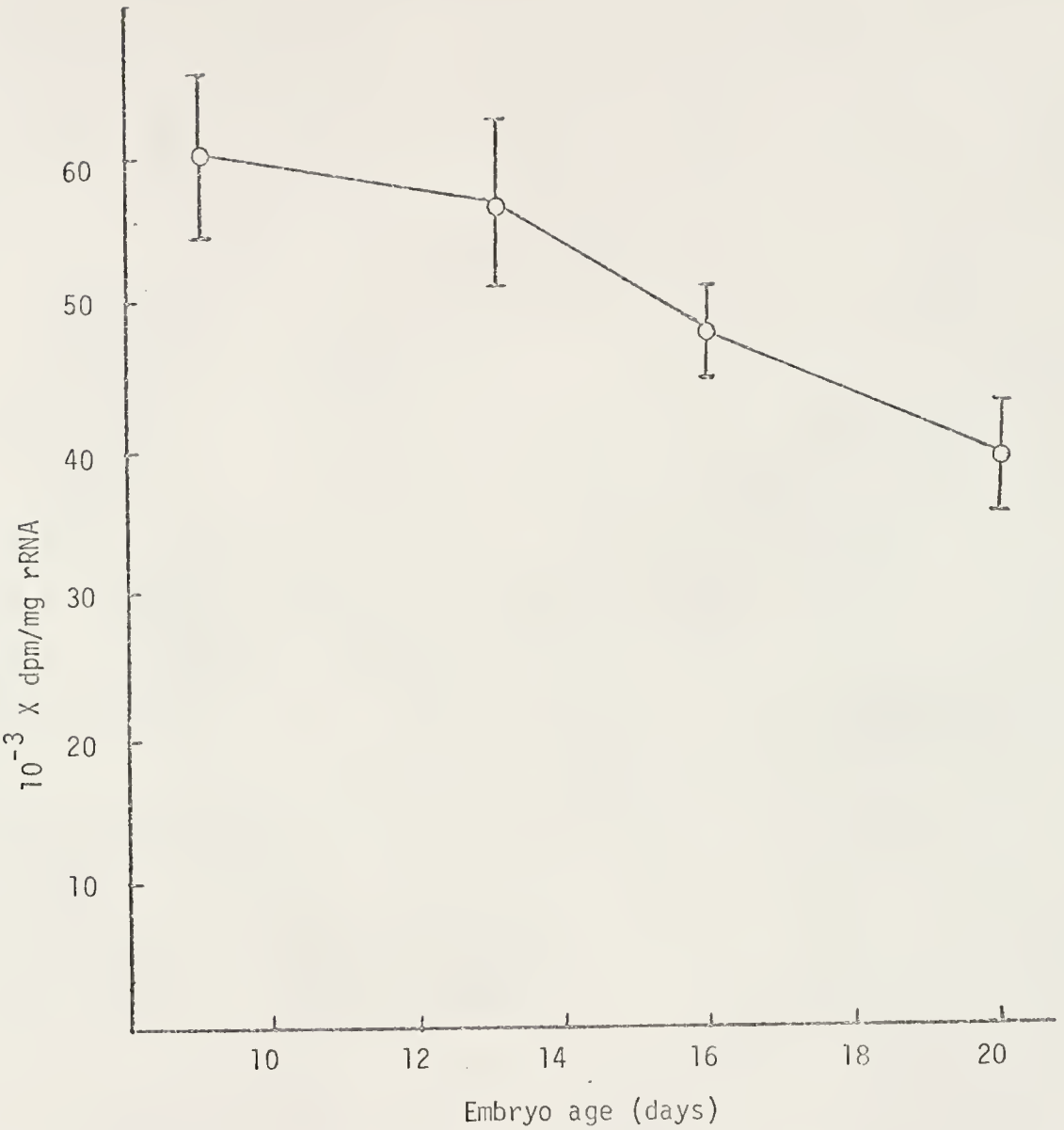


Figure 16. Lysine incorporation from [^{3}H] lysyl-tRNA into polypeptide by cell saps derived from embryos of various ages. Cell saps prepared from embryos of different ages were incubated with a standard salt-washed polysome preparation as described in Materials and Methods. The incorporation was determined using three different cell sap concentrations (between 0.5 and 1.5 mg protein per ml reaction mixture). The results have been normalized to incorporation per mg cell sap protein. Each point represents the mean $\pm$ S.E. of three separate experiments.

Aminoacyl-tRNA Synthetase Activity

The ability of cell sap to activate amino acids was studied. Aminoacyl-tRNA synthetase activity was assayed by the exchange of ^{32}P labeled pyrophosphate with the pyrophosphoryl moiety of ATP in the presence of amino acids. There is some evidence that synthetases are associated with large particles (Hampel and Enger, 1973). The distribution of synthetase activity in both the cell sap and microsomal fraction was studied. As shown in Table 2, while some activity was found in the microsomal fraction, more than 90% of the synthetase activity was found in the cell sap. Preliminary experiments were performed by measuring the synthetase activities of different age embryos using both postmitochondrial supernatant and cell sap. Parallel results were obtained. Therefore, in the following assay cell sap was used as the source of enzyme.

While the pyrophosphate exchange reaction is dependent on the presence of added amino acids (Table 3), there was a high background exchange between pyrophosphate and ATP in the absence of added amino acids. Dialysis of the sample reduced the background considerably. After subtracting the blank without added amino acids, the activity of the sample before and after dialysis was essentially the same.

The time course of pyrophosphate exchange is shown in Figure 17. The reaction was linear with time up to 20 minutes. The exchange reaction is linearly dependent upon the cell sap concentration thus permitting the comparison of synthetase activity under these assay conditions (Figure 18).

Table 2

Subcellular Distribution of Aminoacyl-tRNA Synthetase Activity

The cell fractions were prepared from 19-day embryonic livers by homogenizing livers in 5 volumes of Buffer I containing 250 mM sucrose. Postmitochondrial supernatant and cell sap were prepared as described in Materials and Methods. The microsomal pellet was resuspended in the original volume of Buffer I, 0.1 ml of each fraction was assayed as described in Materials and Methods.

Cell fraction	Synthetase Activity nmole ^{32}PPi incorpd into ATP/15 min
Postmitochondrial supernatant	378
Cell sap	357
Microsomal pellet	32

Table 3

Amino Acid Requirement of the Pyrophosphate-ATP Exchange Reaction

Cell sap prepared from 19-day embryos was divided into two portions. One portion was dialyzed overnight at 4°C against Buffer I containing 250 mM sucrose. The other portion was stored at 4°C overnight. Pyrophosphate-ATP exchange was assayed with and without added amino acid mixture as described in Materials and Methods.

	nmole ^{32}PPi incorpd into ATP/15 min/mg protein
Cell sap	112
Cell sap + amino acid mixture	658
Cell sap (dialyzed)	44
Cell sap (dialyzed) + amino acid mixture	536

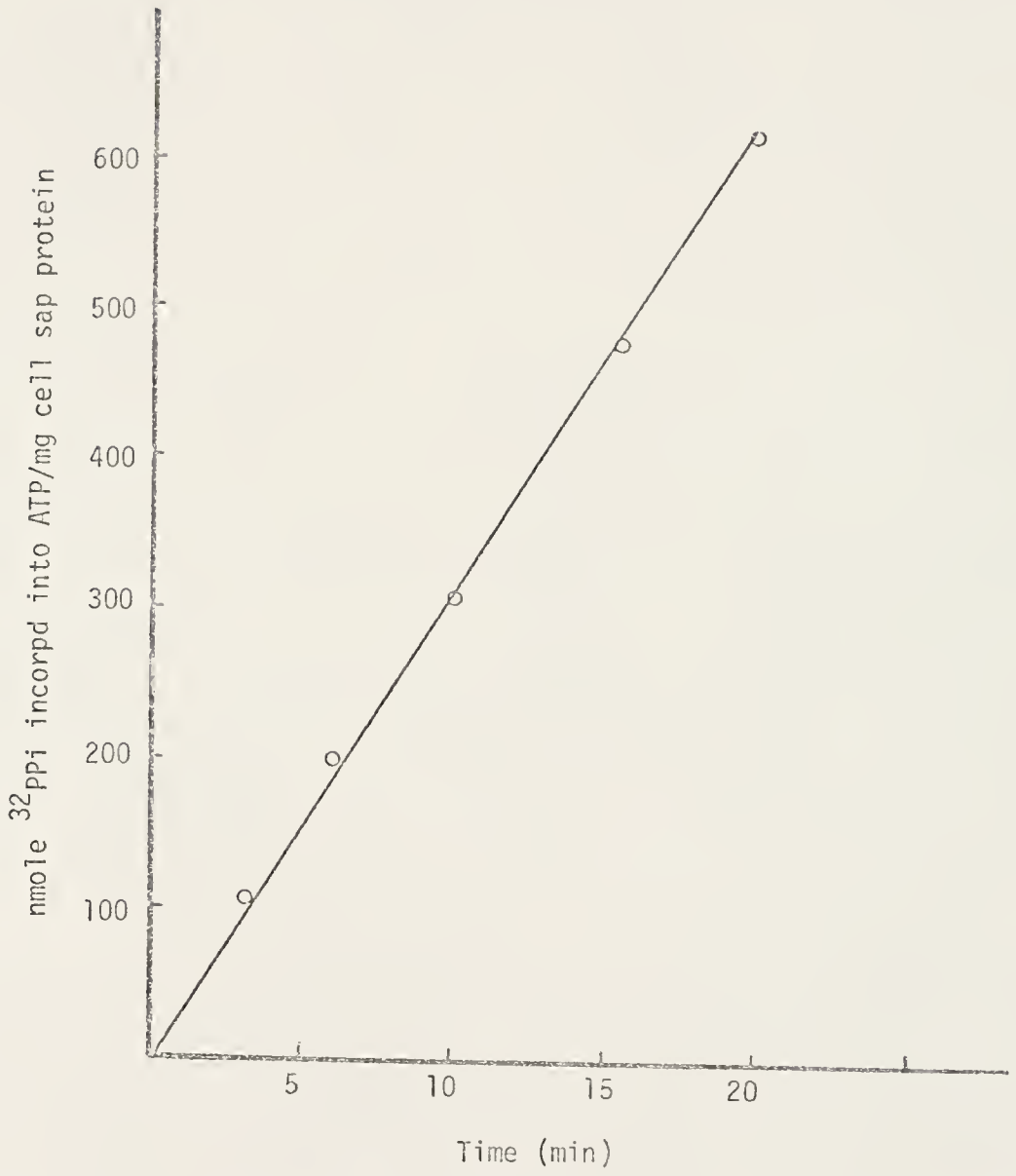


Figure 17. Time course of pyrophosphate-ATP exchange assay. Cell sap was prepared from 17-day embryonic livers and was dialyzed overnight. The reaction conditions have been described in Materials and Methods. Approximately 500 ug cell sap protein/ml reaction mixture was used to determine the time course. Duplicate 1 ml reaction mixtures were assayed at each time interval. Blanks without added amino acid have been subtracted from each point.

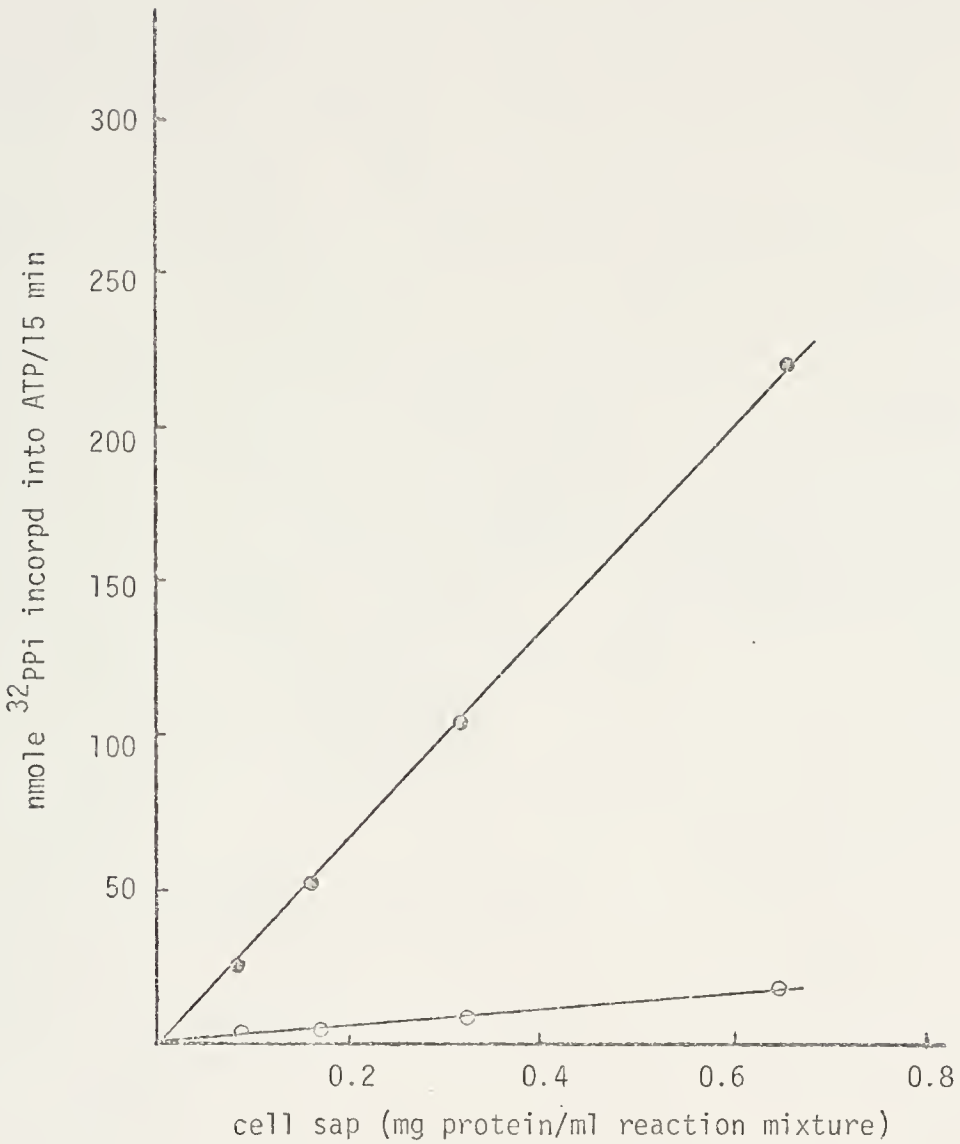


Figure 18. Effect of cell sap concentration on the pyrophosphate exchange reaction. Cell sap was prepared from 17-day embryonic livers and was dialyzed overnight. The reaction conditions have been described in Materials and Methods. Each point represents the average of duplicate determinations. ●, incubation with added amino acid mixture; o, incubation without added amino acid mixture.

Figure 19 shows the variation of aminoacyl-tRNA synthetase activity of cell saps prepared from embryos between 9 and 20 days of development. Synthetase activity seems to be somewhat higher in the cell sap derived from embryos at later stages of development. However, the activity difference throughout the development stages we studied was less than 20%.

From the results, it becomes obvious that the aminoacyl-tRNA synthetase activity in the cell sap can not account for all the decrease in cell sap activity in supporting protein synthesis during development. Based on the results of this experiment and the one on the incorporation of [^{3}H] aminoacyl-tRNA, it seems reasonable to assume that the decrease in cell sap activity is probably due to a cell sap component involved in a stage of protein synthesis different from and possibly subsequent to the aminoacylation of tRNA.

Ribonuclease Activity

Ribonuclease activity in various subcellular fractions of embryonic livers at different stages of development was assayed. The results are summarized in Table 4. Ribonuclease activity in whole liver homogenate almost doubled between 8 and 15 days of development. The particulate fraction contained most of the cellular ribonuclease activity. The changes in activity of the particulate fraction with development paralleled those of the whole cell homogenate. Before the 12th day, no ribonuclease activity could be detected in the cell sap. However, this activity increased 8-fold from day 12 to day 19. Some activity was present in the microsomal fraction and it increased 5-fold from day 8 to day 19.

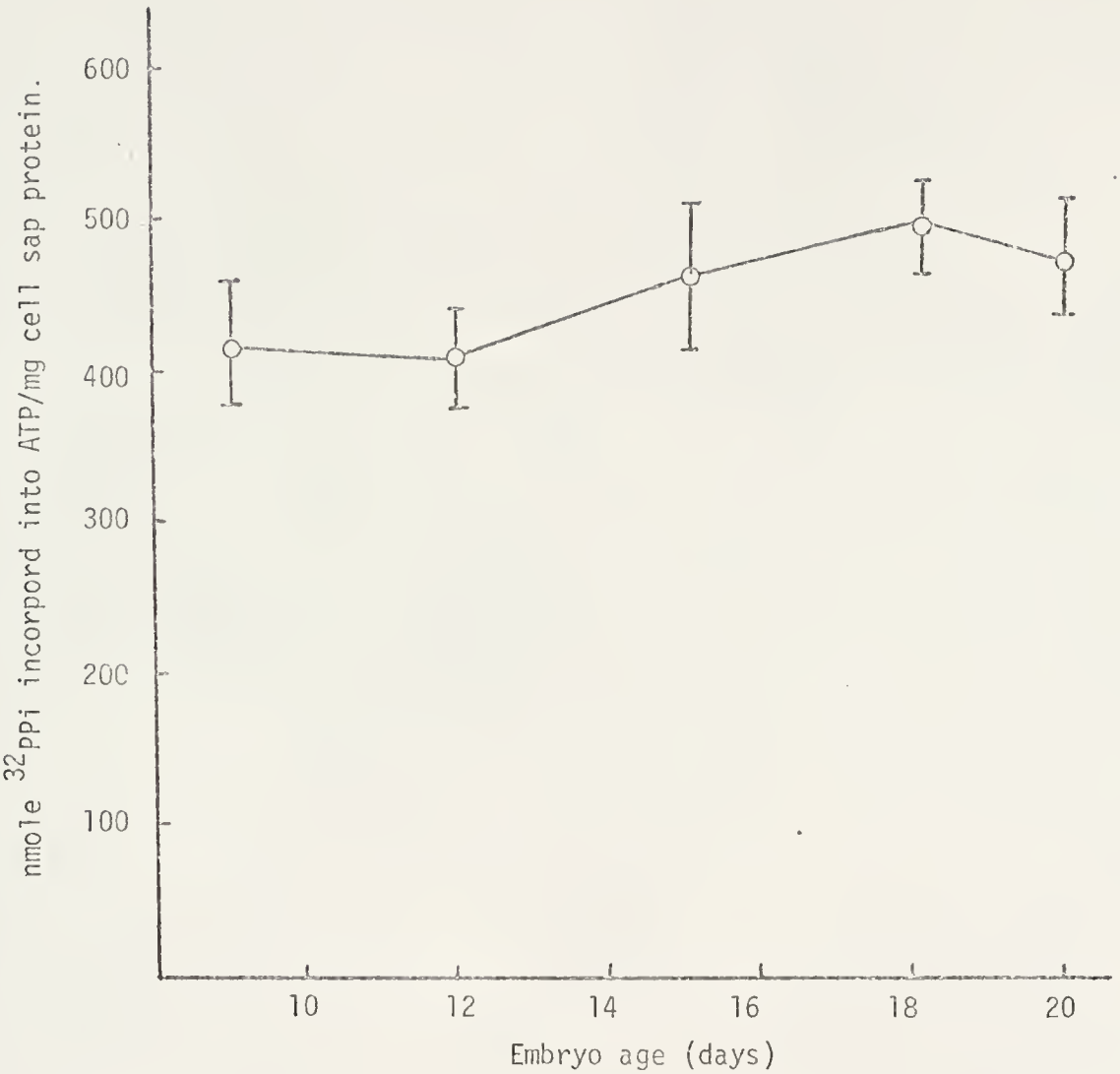


Figure 19. Aminoacyl-tRNA synthetase activity of cell sap derived from embryos of various ages. The cell saps were dialyzed overnight and pyrophosphate-ATP exchange assayed was performed as described in Materials and Methods. Incubation was for 15 min at 35°C. The values represent the mean ± S.E. of 3 to 5 separate experiments.

Table 4

Ribonuclease Activity in Liver Subcellular Fractions Derived from Embryos of Various Ages

Livers were homogenized in 9 volumes of Buffer I containing 250 mM sucrose. The homogenate was centrifuged at 12,000 X g_{max} for 15 min. The pellet was resuspended in the same volume of Buffer I containing 250 mM sucrose and designated "particulate fraction." Cell sap and microsomal fractions were prepared from the supernatant. Ribonuclease activity was assayed as described in Materials and Methods. The results are arbitrarily expressed as the difference in A_{260} between samples and blanks. The values shown have been normalized to relative ΔA_{260} per gram liver.

Embryo age (days)	ΔA_{260}			
	Homogenate	Particulate fraction	Microsome	Cell sap
8	2.30	2.19	0.031	0.015
12	3.11	3.27	0.078	0.011
15	4.15	4.01	0.110	0.067
19	4.08	4.55	0.159	0.128

Measurement of cell sap inhibitor activity against bovine pancreatic ribonuclease showed that no inhibition could be detected in the cell sap derived from embryos before 15 days of age (Table 5). Twenty to thirty percent inhibition was observed after day 17. Similar levels of inhibition were seen when the inhibitor activity was assayed against the endogeneous ribonuclease in the liver particulate fraction. The amount of inhibition observed in the older embryos was barely within the range of significance. Many mechanisms could be responsible for this small decrease in ribonuclease activity. One would be the presence of a specific inhibitor. Another possible explanation would be that such inhibition was nonspecifically caused by the adsorption of added ribonuclease to some cell sap component. The third reason may be that the assay method is not sensitive enough to detect changes in the endonuclease activity. Since the amount of substrate RNA used in the assay was in large excess, it is less likely that the inhibition was due to competition for the substrate. The results are in line with that of Roth (1962). He reported 30-60% inhibition for pancreatic ribonuclease by adult chicken liver high speed supernatants under similar assay conditions, but no inhibition by supernatant from 8-day and 9-day whole embryos.

Increase in cellular protein synthesis has been demonstrated to be associated with decreased ribonuclease activity and/or increased ribonuclease inhibitor activity, and vice versa (Shortman, 1962; Arora and de Lamirande, 1967; Moriyama *et al.*, 1969; Sheppard *et al.*, 1970). It seems possible that the increase in ribonuclease activity of cell sap with increasing developmental age may be connected with the decrease in amino acid incorporating activity of the cell sap.

Table 5

Ribonuclease Inhibitor Activity in Cell Sap Derived from Embryos of Various Ages

Livers were homogenized in 9 volumes of Buffer I containing 250 mM sucrose. The reaction mixture in 1 ml contained 0.0125 µg bovine pancreatic ribonuclease or 0.1 ml particulate fraction (resuspended in same original volume), 0.4 ml cell sap, 0.4 ml of 1% yeast RNA and 0.1 ml of 200 mM Tris-HCl (pH 7.4). The mixture was incubated at 35°C for 60 min and precipitated with 0.5 ml of 0.75% uranyl acetate in 25% $HClO_4$ and treated as described in Materials and Methods. Each value represents mean ± S.E.

Embryo age (days)	% inhibition	
	Pancreatic RNase	Particulate Rnase
8	2 ± 4	-
12	4 ± 3	6 ± 4
15	10 ± 4	-
17	21 ± 6	-
19	27 ± 5	25 ± 8
20	24 ± 8	-

Distribution and Activity of Free and Membrane-Bound Polysomes

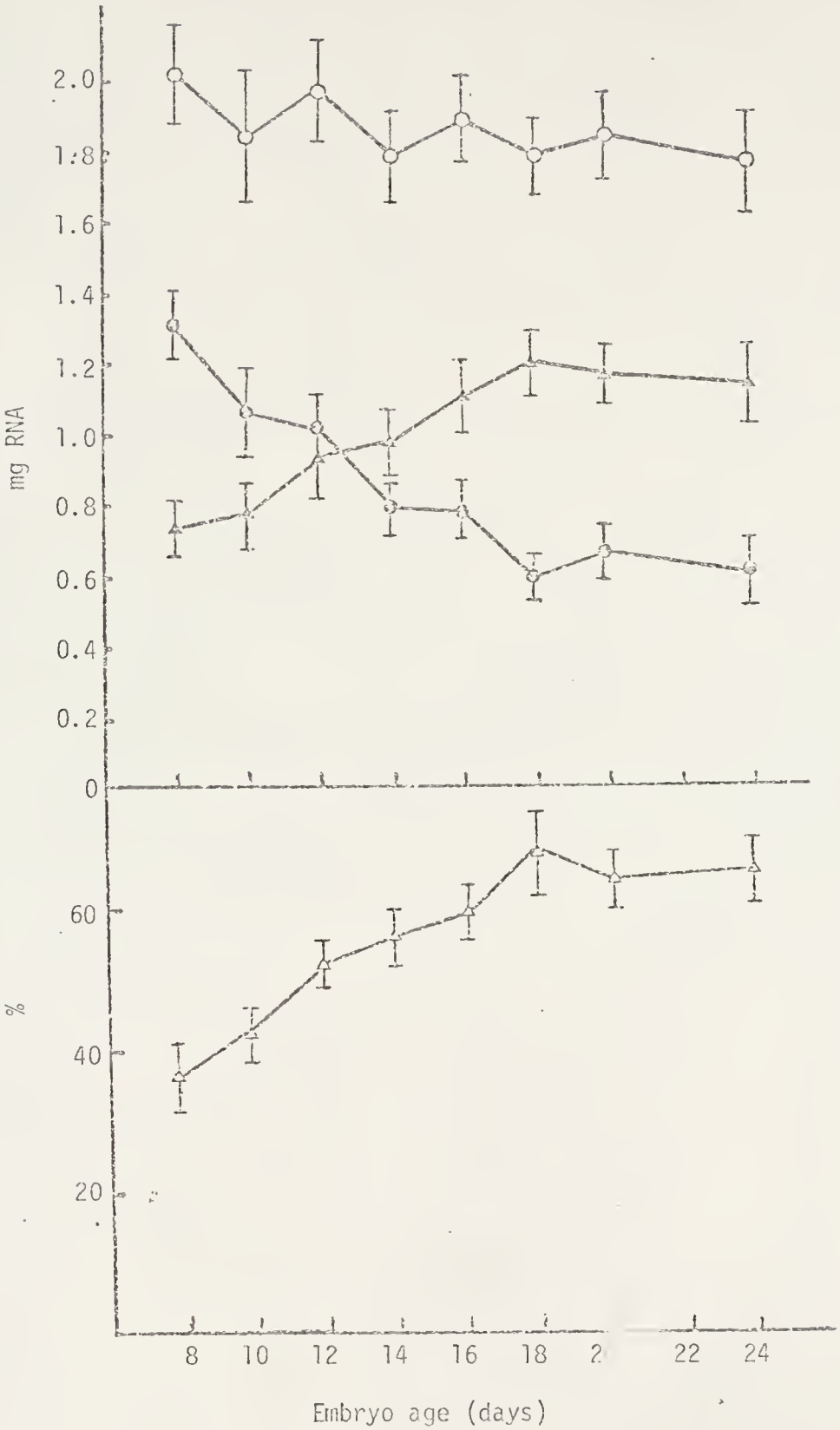
Distribution of Free and Membrane-Bound Polysomes

Since serum proteins are probably synthesized by membrane-bound polysomes and early embryonic liver cells contain very little endoplasmic reticulum, we looked for a correlation between the distribution of membrane-bound and free polysomes and changes in protein synthetic capacity during development. The amount of membrane-bound and free polysomes was estimated by a modification of the method of Blobel and Potter (1967). Because a significant amount of membrane-bound polysomes tends to sediment in the mitochondrial fraction, total cytoplasmic polysomes and free polysomes are separated from postnuclear supernatant. Membrane-bound polysome content was determined as the difference between total and free polysome content thus circumventing incomplete recovery of membrane-bound polysomes from the boundary of two sucrose layers. Usually the nuclear fraction contained 7-10% of the total cellular RNA.

The total polysome content per gram of liver at various ages was relatively constant (Figure 20). The free polysome content decreased markedly between day 8 and day 18, whereas the membrane-bound polysome content increased during the same period. Hence the percentage of total polysomes present on membranes increased 2-fold as development progressed.

To ascertain that ribosomes did not readsorb the membrane during isolation, a mixing experiment was performed. Postnuclear supernatant was prepared from a 1:1 mixture of 10-day and 17-day liver homogenates. Free and membrane-bound polysome contents were measured in this mixture

Figure 20. Distribution of free and membrane-bound polysomes in livers at various stages of development. Livers were homogenized in 3 volumes of Buffer I containing 250 mM sucrose. Free and membrane-bound polysomes were fractionated from the postnuclear supernatant as described in Materials and Methods. The values are the means ± S.E. of 3 to 4 separate experiments. The results were expressed in mg RNA per ml homogenate. o, total polysomes; ●, free polysomes; ▲, membrane-bound polysomes; △, % membrane-bound in total polysomes.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
mg RNA
60
40
20
%
8
10
12
14
16
18
22
24
Embryo age (days)

as well as in the 10-day and 17-day controls. The amount of free and membrane-bound polysomes found in the mixture was approximately the same as the average of the two controls (Table 6). Therefore, reassociation of free ribosomes with the membrane during isolation was not significant.

These experiments indicate that the proportion of membrane-bound polysomes in total polysomes increases during development. Chemical measurements by Pollak and Ward (1967) showed that the phospholipid content of the microsomal fraction increases during development. An increase in rough endoplasmic reticulum seems to be a general phenomenon in cells undergoing rapid growth such as in the neonatal (Dallner *et al.*, 1966) and regenerating rat liver (Tata, 1970). The exact function of the ribosome-membrane assembly is not clear, since an accumulation of intracellular protein also occurs during the same period. Because of the increase in serum protein concentration in blood during development, it seems highly probable that the increase in membrane-bound polysomes in embryonic chick liver results in the increased synthesis of secreted proteins.

Activity of Free and Membrane-Bound Polysomes

The preceding experiments suggest that the distribution of free and membrane-bound polysomes changes while the amino acid incorporating activity of total polysomes remains the same during development. It was not known whether the activity of the free and membrane-bound polysomes is the same or whether their activities change during development. To resolve this question, the amino acid incorporating activity of these two types of polysomes was assayed.

Table 6

Distribution of Free and Membrane-Bound Polysomes in Homogenates of 10-day and 17-day Mixture

Livers were homogenized in 3 volumes of Buffer I containing 250 mM sucrose. Postnuclear supernatant was prepared from 1:1 mixture of 10-day and 17-day liver homogenates. Free and membrane-bound polysomes were fractionated as described in Materials and Methods. The values represent the average of 3 determinations in a single experiment. The results were expressed in mg RNA in 1 ml homogenate.

Embryo age days	Total polysomes mg RNA	Free polysomes mg RNA	Membrane-Bound polysomes mg RNA	Bound/Total polysomes %
10	1.92	1.13	0.79	41
17	1.81	0.63	1.18	65
10 + 17	1.88	0.92	0.96	51

For studying amino acid incorporation, membrane-bound and free polysomes were isolated from postmitochondrial rather than postnuclear supernatant, because polysomes were more degraded in the postnuclear supernatant. The disadvantage of the procedure used is that considerable losses of membrane-bound polysomes occur during separation of postmitochondrial supernatant.

The amino acid incorporating activity of free and membrane-bound polysomes prepared from 12-day, 15-day and 19-day embryonic livers is shown in Table 7. Because of the small size and low membrane-bound polysome content of liver, polysome analyses from embryos younger than 12 days were not significant. The activity of free polysomes derived from embryos of these three ages was approximately the same. In contrast, the activity of membrane-bound polysomes appeared to decrease with age. The activity of free polysomes was found to be higher than that of membrane-bound polysomes derived from embryos of the same age. The difference between the activities of the two types of polysomes was more pronounced in the older embryos compared with that in young embryos. However, polysomes could be released from the 19-day membrane-bound polysome preparation by detergent treatment (Figure 21). The greater amount of ultraviolet absorbing material in the upper fractions of the membrane-bound versus free polysome preparations is probably membranous material released upon detergent treatment. The time course of incorporation showed that these two types of polysomes incorporated amino acid at the same initial rate, however; with membrane-bound polysomes it leveled off earlier than that with

Table 7

Amino Acid Incorporating Activity of Free and Membrane-Bound Polysomes Before and After Triton X-100 Treatment

Free and membrane-bound polysomes were prepared and assayed as described in Materials and Methods. Incubation was for 30 min. The values represent mean ± S.E. from 3 to 4 experiments.

Embryo age days	Before Triton X-100 Treatment			After Triton X-100 Treatment		
	Free polysomes	Bound polysomes	Free/Bound	Free polysomes	Bound polysomes	Free/Bound
	pmole [^{3}H] lysine incorpd/mg rRNA		%	pmole [^{3}H] lysine incorpd/mg rRNA		%
12	423 ± 36.1	348 ± 31.8	82	360 ± 24.9	327 ± 35.4	91
15	396 ± 28.8	285 ± 23.7	72	354 ± 36.7	345 ± 28.5	97
19	384 ± 52.2	204 ± 48.9	53	318 ± 39.6	336 ± 45.1	106

Figure 21. Sucrose gradient profiles of membrane-bound and free polysomes prepared from 19-day embryonic livers without using detergent. Polysomes were analyzed after addition of detergent to the membrane-bound polysome preparation as described in Materials and Methods. Approximately 3.0 A_{260} units of polysomes were layered over 36 ml 15-40% sucrose gradients centrifuged and monitored as described. ———, represents free polysomes; — — —, represents membrane-bound polysomes.

0.4
0.3
0.2
0.1
A_{254}
10
20
30
ml from meniscus

free polysomes (Figure 22). Once again the difference was greater in older embryos.

In the above experiment, the two types of polysomes were prepared without using detergent. Because the membrane was still associated with polysomes during the assay of amino acid incorporation, the observed difference in activity could not be attributed solely to the polysome. In other words, the presence of membrane may be inhibitory to amino acid incorporation. To test this possibility, both free and membrane-bound polysomes were treated with Triton X-100 and reisolated. To obtain undegraded polysomes, yeast RNA had to be added to the polysome preparation during detergent treatment. In the absence of yeast RNA, membrane-bound polysomes were degraded extensively as shown by the sucrose gradient profiles in Figure 23. Unlike the finding with rat liver (Blobel and Potter, 1966), addition of cell sap to the membrane-bound polysomes during Triton X-100 treatment did not protect polysomes from degradation (Figure 23B). In contrast, relatively undegraded free polysomes were isolated in the absence as well as the presence of yeast RNA (Figure 24). It appeared that the detergent treatment might release or activate some ribonuclease present in the polysome preparations. Therefore, ribonuclease activity in the polysome preparations was assayed (Table 8). Before Triton treatment, the ribonuclease activity in free polysomes from 18-day embryos was low (just twice the detectable level in this assay). That in the membrane-bound polysomes was 5-fold higher. After membrane was removed by detergent, the polysomes originally bound to the membrane contained one third their original ribonuclease activity. Since more ribonuclease activity

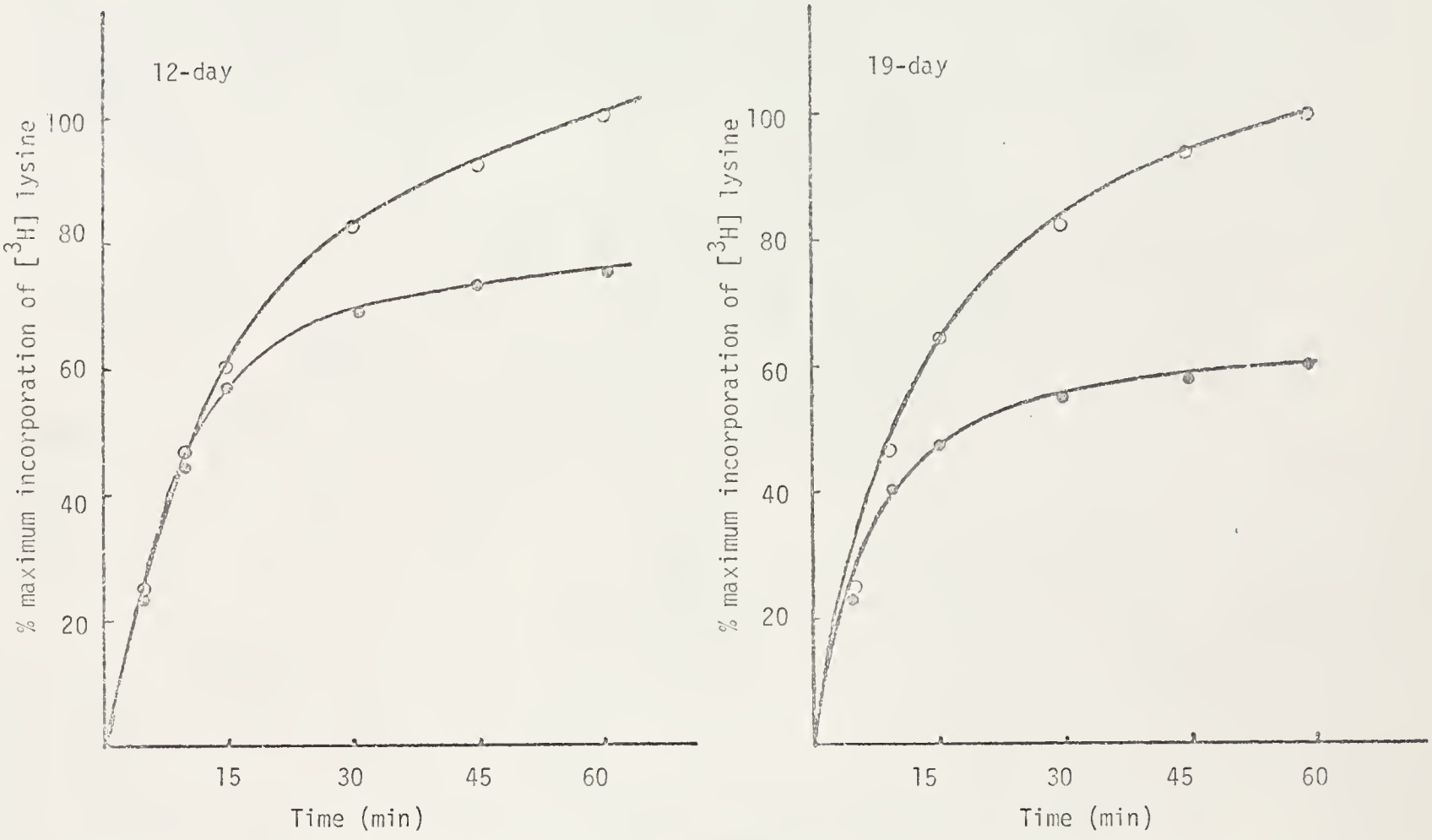


Figure 22. Time courses of amino acid incorporation of membrane-bound and free polysomes. Membrane-bound and free polysomes were prepared from 12-day and 19-day embryonic livers without using detergent. Polysomes were incubated with homologous cell sap and assayed as described in Materials and Methods. Incorporation values were expressed as % maximum of free polysomes at 60 min. Separate maximum incorporation values were used for 12-day and 19-day preparations. o, free polysomes; ●, membrane-bound polysomes.

Figure 23. Sucrose gradient profiles of membrane-bound polysomes after Triton X-100 treatment. Membrane-bound polysomes prepared from 19-day embryonic livers (approximately 20 A_{260} units/ml) were divided into three portions. Each portion was treated with 20% Triton X-100 to a final concentration of 1% (A) in the presence of 20 A_{260} units/ml low molecular weight yeast RNA (B) in the presence of cell sap (approximately 3 mg protein/ml) (C) in the absence of yeast RNA and cell sap. Each portion was layered over 2 ml of 1.0 M sucrose in Buffer I and centrifuged at 226,000 X g_{max} for 2.5 hrs to resediment polysomes. The pellets were resuspended in Buffer I and approximately 4.0 A_{260} units of each sample were layered on a 36 ml 15-40% sucrose gradient, centrifuged and monitored as described.

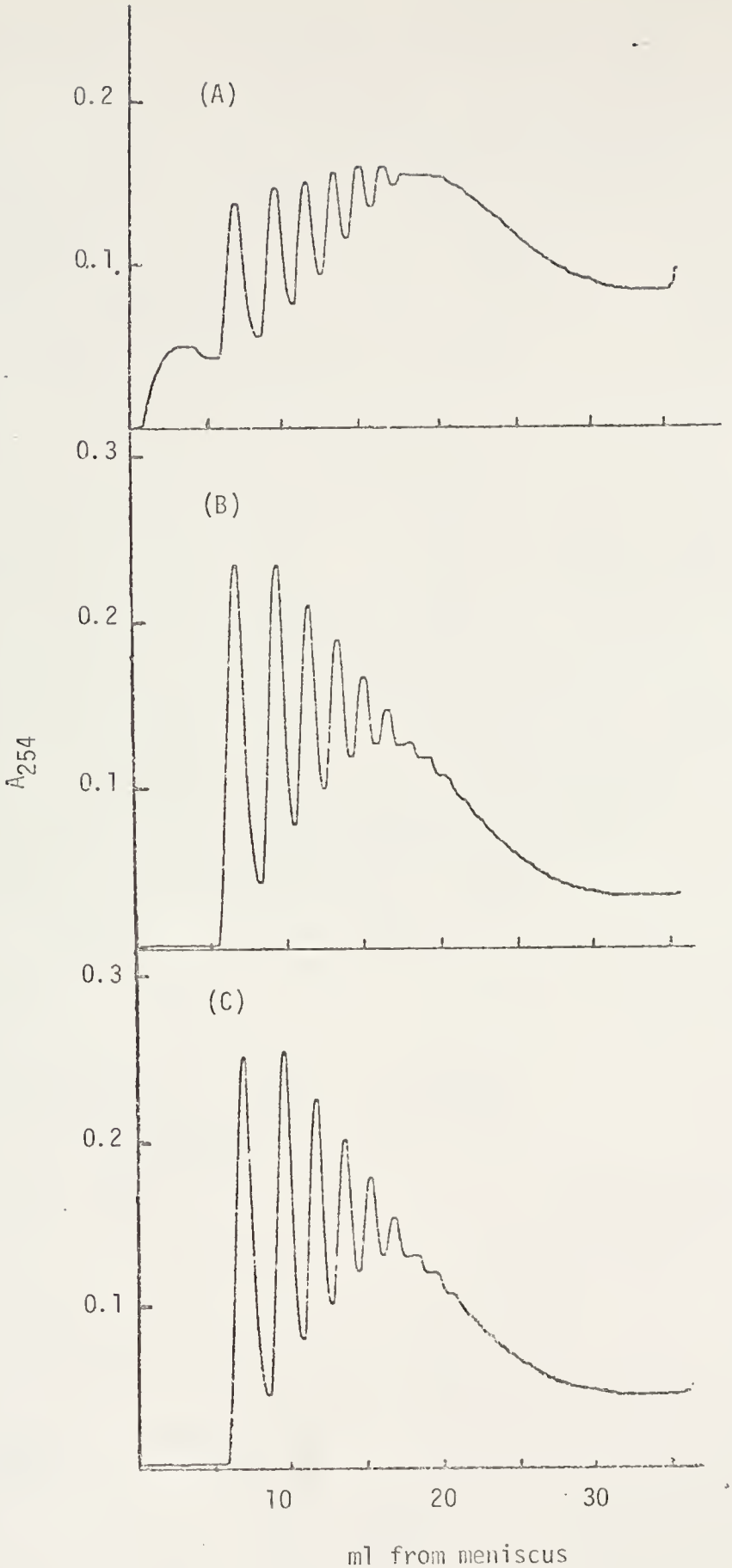

(A)
(B)
(C)
0.2
0.1
0.3
0.2
0.1
0.3
0.2
0.1
A_{254}
10
20
30
ml from meniscus

Figure 24. Sucrose gradient profiles of free polysomes after Triton X-100 treatment. Free polysomes prepared from 19-day embryonic livers (approximately 20 A_{260} units/ml) were divided into 2 portions. Each portion was treated with 20% Triton X-100 to a final concentration of 1% (A) in the presence of 20 A_{260} units/ml of low molecular yeast RNA (B) in the absence of yeast RNA. Each sample was layered over 2.0 ml of 1.0 M sucrose in Buffer I and centrifuged at 226,000 X g_{max} for 2.5 hrs to resediment polysomes. The pellets were resuspended in Buffer I and approximately 6.0 A_{260} units of each sample were layered on a 36 ml 15-40% sucrose gradient and centrifuged and monitored as described.

(A)
0.3
0.2
0.1
A_{254}
(B)
0.3
0.2
0.1
10
20
30
ml from meniscus

Table 8

Ribonuclease Activity in Free and Membrane-Bound Polysomes

Free and membrane-bound polysomes were prepared as described in Materials and Methods. Membrane-bound polysomes were treated with 20% Triton X-100 to a final concentration of 1%. The mixture was layered over 2.0 ml of 1.0 M sucrose in Buffer I and centrifuged at 226,000 X g_{max} for 2.5 hours to sediment polysomes. The supernatant was diluted 4 times and centrifuged at 226,000 X g_{max} for 1 hour. The pellet thus obtained was resuspended in Buffer I of the same volume as the original membrane-bound polysomes preparation and designated "membranous material." Ribonuclease activity was assayed as described in Materials and Methods. Incubation was at 35°C for 60 min.

	12-day embryo	18-day embryo
	ΔA_{260}/mg rRNA	
Before Triton X-100 treatment		
Free polysomes	0.043	0.061
Membrane-bound polysomes	0.144	0.306
After Triton X-100 treatment		
Membrane-bound polysome	0.072	0.093
Membranous material	-	0.401

was found in the membranous material than was present with the membrane-bound polysomes, the detergent appears to enhance the nuclease activity. Similar results were obtained for polysomes derived from 12-day embryos but the nuclease activity was much lower in all fractions as compared with 18-day embryos. The results suggest that the ribonuclease activity found in polysome preparations is associated with the membrane rather than with the polysome. The small degree of degradation of free polysomes upon detergent treatment shown in Figure 24 may be due to some membrane contamination in the free polysome preparations.

The A_{260}/A_{280} ratios of the Triton X-100 treated free and membrane-bound polysomes were 1.75-1.88 which indicated that the polysomes were almost completely devoid of membrane. The amino acid incorporating activity of the detergent treated polysomes is shown in Table 7. No difference in activity was observed between free and membrane-bound polysomes derived from embryos of the ages tested. This result suggests that the activity difference observed between free and membrane-bound polysomes before detergent treatment is probably due to some component present in the membrane. Ribonuclease activity found in the membrane may be that one component. The more rapid decay of amino acid incorporating activity of membrane-bound polysomes as compared with that of free polysomes is consistent with a membrane-bound nuclease active during incubation for amino acid incorporation. The higher nuclease activity in the membrane-bound polysomes of older embryos could also explain the relative activity difference between membrane-bound and free polysomes. It is interesting to note that detergent treatment has been shown to alter the behavior of membrane-bound polysomes by other workers

(Bloemendal *et al.*, 1967; McDonald and Konner, 1971). However, the difference may also be ascribed to a different average size of the mRNA molecules in the two polysomes.

The relative amino acid incorporating activity of membrane-bound and free polysomes seems to depend upon the method as well as the ionic conditions used for isolation of the two polysome fractions, since in rat liver cells, membrane-bound polysomes have been reported to be more (Tata and Williams-Ashman, 1967; Pain *et al.*, 1974), equally (Bloemendal *et al.*, 1967; Takagi and Ogata, 1968), or less (Ragnotti, Lawford and Campbell, 1969; McDonald and Konner, 1971) active than free polysomes, apparently the result of different isolation procedures. Under the assay conditions described above, we detected no difference between the activity of free and membrane-bound polysomes. However, membrane seems to affect amino acid incorporation by polysomes in the cell-free system.

Albumin Synthesis

As a first step in our study of the molecular events that initiate and control albumin synthesis during development, it was decided to measure albumin synthesis in the cell-free system. Free and membrane-bound polysomes were incubated for amino acid incorporation. After incubation, the reaction mixtures were centrifuged to sediment polysomes. Approximately 30-40% of the hot trichloroacetic insoluble counts was released into the supernatant from the free polysomes and 15-20% of counts was released from the membrane-bound polysomes. The supernatants from each reaction mixture were dialyzed extensively and

then subjected to gel electrophoresis at pH 8.9 in a 7.5% polyacrylamide gel. Under these conditions, authentic chicken serum albumin migrates faster than most other serum proteins so that it appears as a discrete band behind the bromphenol blue tracking dye. Figure 25 shows the gel patterns of the dialyzed supernatants derived from 16-day embryos. No significant radioactive peak appeared to migrate at the position of standard albumin. Finding no albumin in the supernatant, the polysomal pellets were resuspended in buffer, frozen, thawed, sonicated and centrifuged again. About 25-30% of the hot trichloroacetic acid insoluble radioactivity was released into the supernatant by such treatment. The released material was dialyzed and analyzed by gel electrophoresis. Figure 26 shows the gel pattern of the materials released from the free and membrane-bound polysomes derived from 16-day embryos. In the free polysome preparation, some radioactivity was present in the region corresponding to albumin standard, but no discrete band was observed. One major peak of radioactivity from the membrane-bound polysome preparation comigrated with standard albumin. To characterize this peak further, carrier chicken serum albumin was added to another aliquot of the sample and the mixture was reacted with goat antiserum against chicken serum albumin. After immunoprecipitation, the supernatant was subjected to gel electrophoresis. The antiserum removed the peak of radioactivity which had comigrated with albumin (Figure 27). The immunoprecipitate was washed several times, dissolved, denatured with SDS and subjected to SDS polyacrylamide gel electrophoresis (Figure 28). A radioactive band was observed comigrating with standard albumin run on a sister gel. The radioactivity in the band

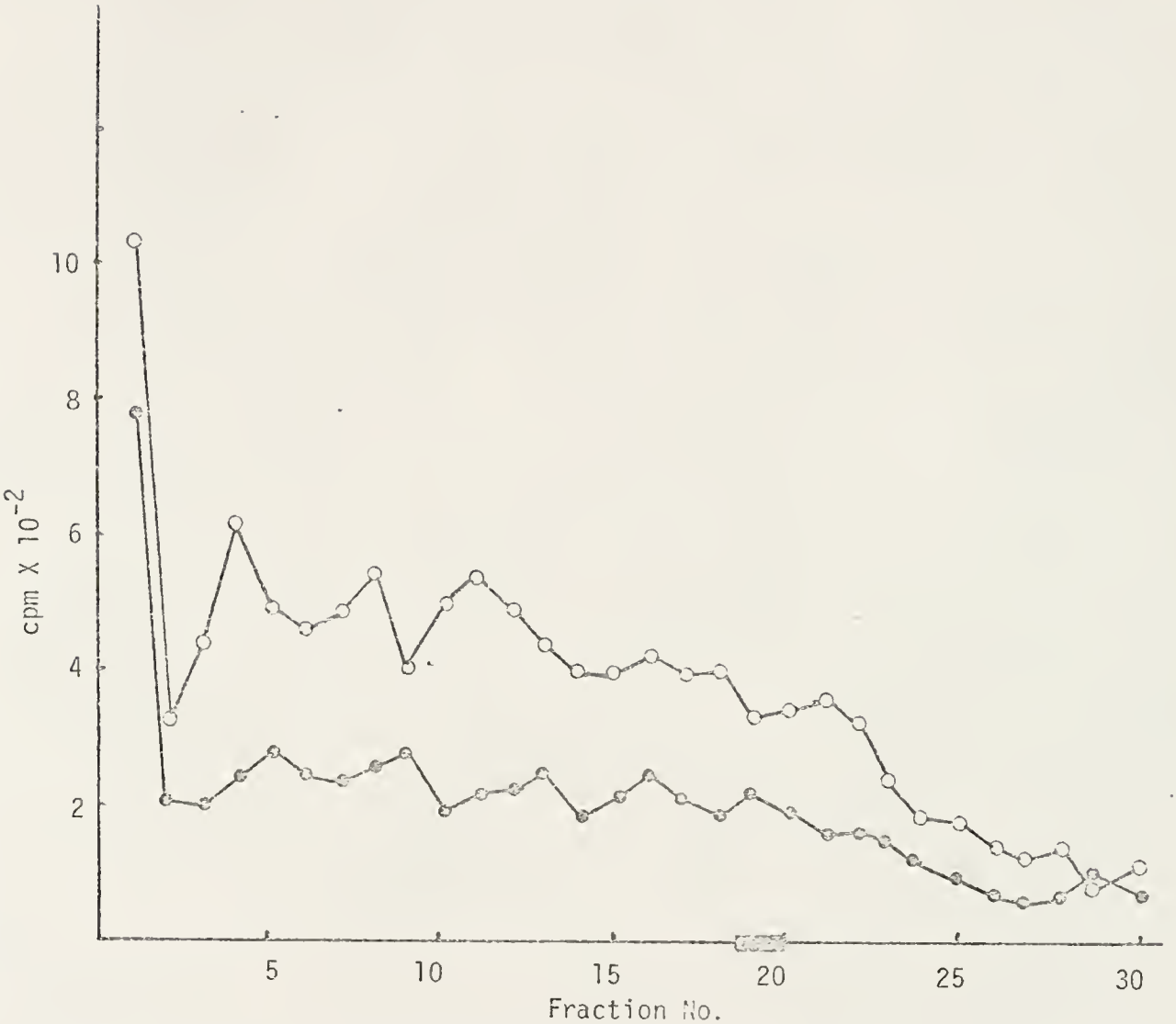
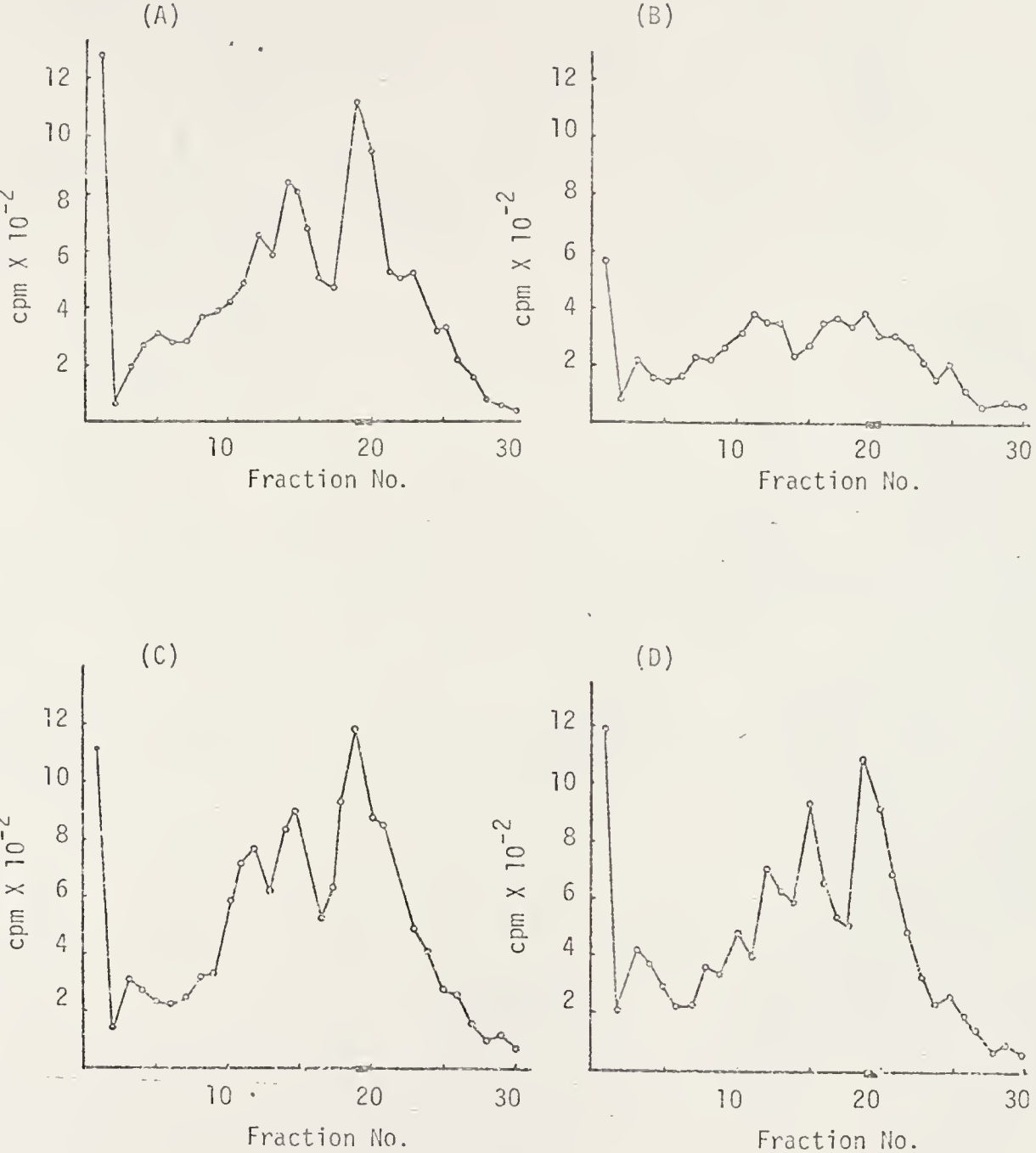


Figure 25. Polyacrylamide gel electrophoresis of cell-free protein products released from membrane-bound and free polysomes before sonication. Membrane-bound and free polysomes prepared from 16-day embryonic livers were incubated for protein synthesis as described in Materials and Methods. After incubation, the reaction mixture was centrifuged to sediment polysomes and the supernatant was dialyzed extensively and analyzed by gel electrophoresis as described in Materials and Methods. Approximately 9000 cpm and 5000 cpm of trichloroacetic acid insoluble material from free and membrane-bound polysomes respectively were applied to the gels. The protein staining band of standard albumin is indicated in the graph. o, free polysomes, ●, membrane-bound polysomes.

Figure 26. Polyacrylamide gel electrophoresis of cell-free reaction products released from membrane-bound and free polysomes by sonication. Conditions for amino acid incorporation and analysis of the products have been described in Materials and Methods. About 7,000 to 15,000 cpm of trichloroacetic acid insoluble material was applied to the gels. The protein staining band of standard albumin is indicated in the graph. A) 16-day membrane-bound polysomes (B) 16-day free polysomes (C) 12-day membrane-bound polysomes (D) 19-day membrane-bound polysomes.


(A)
(B)
(C)
(D)
cpm X 10^{-2}
Fraction No.
2
4
6
8
10
12
10
20
30

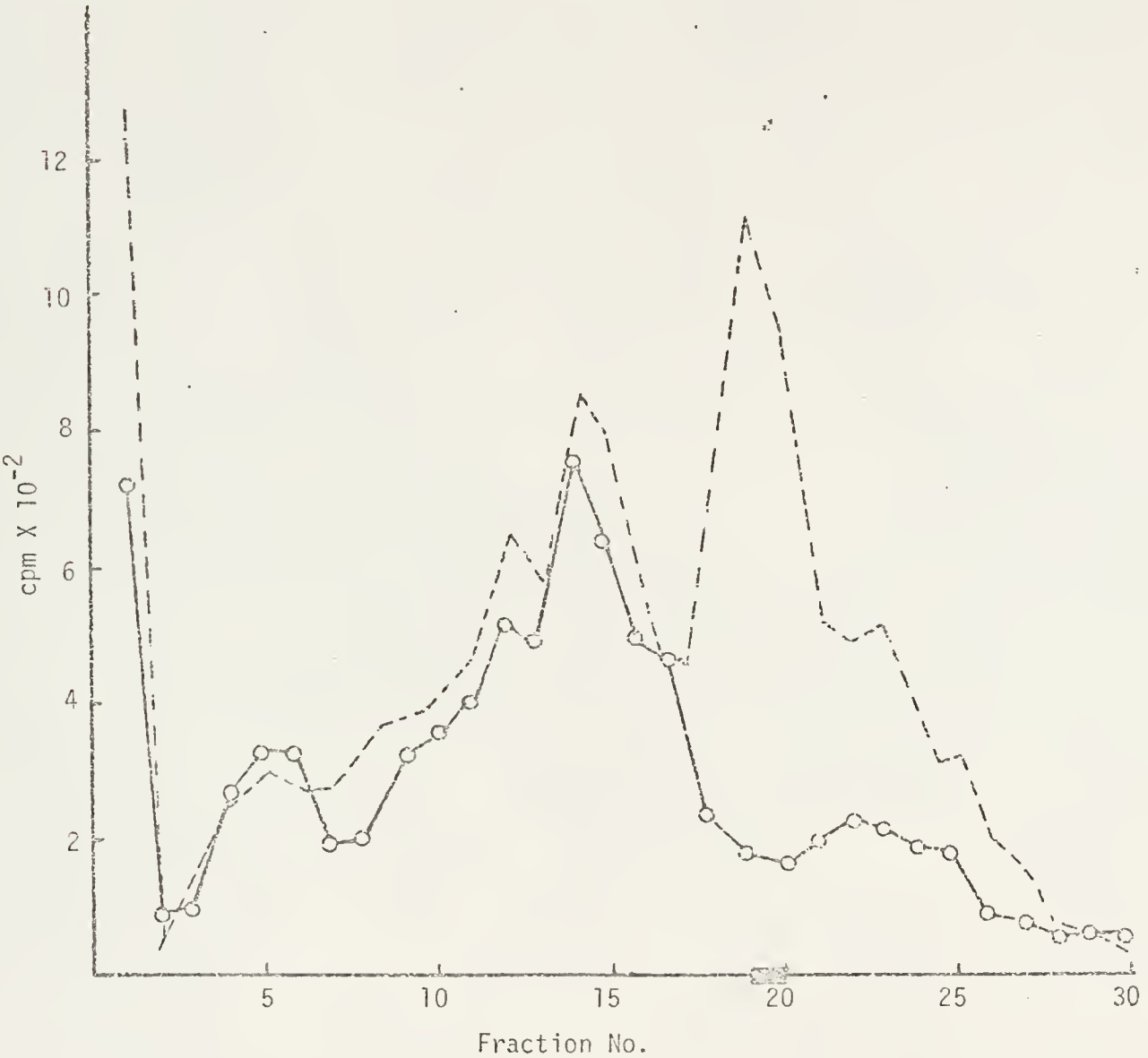


Figure 27. Polyacrylamide gel electrophoresis of cell-free protein products of 16-day membrane-bound polysomes remaining after precipitation with antiserum against albumin. The cell-free protein products were incubated with antiserum against albumin. After removal of the immunoprecipitate by centrifugation, the supernatant was subjected to gel electrophoresis as described in Materials and Methods. The dotted line is the profile of the radioactive products present prior to immunoprecipitation. The protein staining band of standard serum albumin is indicated in the graph.

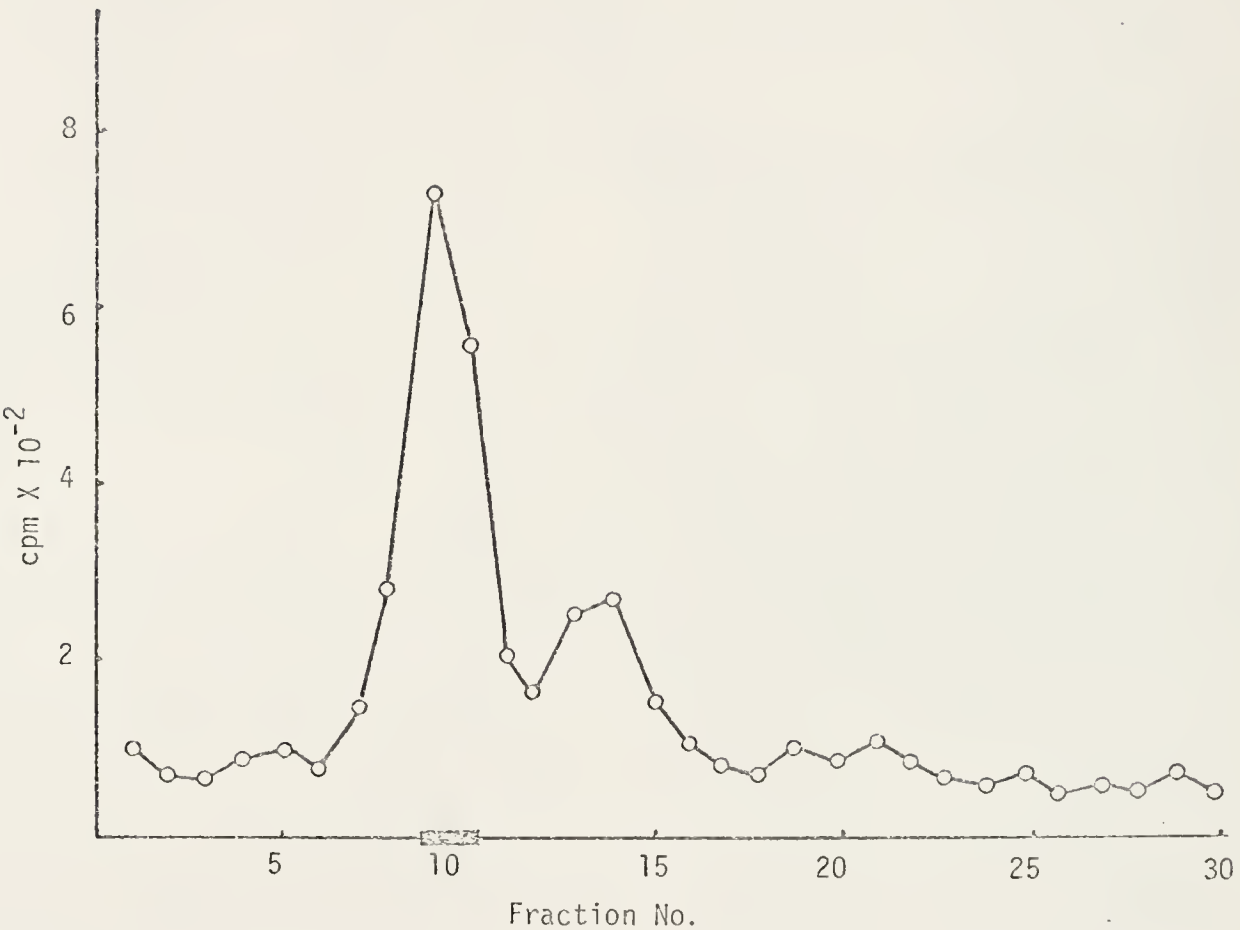


Figure 28. SDS-polyacrylamide gel electrophoresis of the cell-free protein products precipitated with antiserum against albumin. The cell-free products from 16-day membrane-bound polysomes were incubated with antiserum against albumin. The immunoprecipitate was washed and dissolved in 20 mM Tris-HCl (pH 7.4), 1% SDS and 1% β-mercaptoethanol. The sample was subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The protein staining band of standard serum albumin is indicated in the graph.

represents more than 70% of total radioactivity recovered from the gel. There was some radioactivity present in a lower molecular weight region which may represent either incomplete albumin chains or non-specific precipitation. These experiments indicate that the major peak of radioactivity represents newly synthesized albumin. The gel patterns of products synthesized by membrane-bound polysomes derived from 12-day and 19-day embryos are shown in Figure 26. They are very similar to the 16-day pattern.

To determine the amount of albumin synthesized, each sample was reacted with antiserum against albumin and the resulting immunoprecipitate was counted. Table 9 summarizes the results. The percentages of albumin in total polypeptides synthesized by membrane-bound polysomes were approximately the same at different ages. The amount of albumin synthesized by free polysomes was much less than that synthesized by membrane-bound polysomes. The result suggests that membrane-bound polysomes are the major site for albumin synthesis in embryonic chick liver cells. It is interesting that this phenomenon is seen even in the 12-day embryonic livers which contain relatively fewer membrane-bound polysomes. The 8- to 10-fold difference in albumin synthesis by membrane-bound versus free polysomes is in the same range as the results of similar investigations in mammalian systems (Shafritz, 1974b; Koga and Tamaoki, 1974).

Although the amount of albumin synthesized per weight of membrane-bound polysomes remained constant during development, the possibility remains that the increase in albumin synthesis *in vivo* is due to the increase in the content of membrane-bound polysomes during development.

Table 9

Albumin Synthesis by Membrane-Bound and Free Liver Polysomes Derived from Embryos of Various Ages

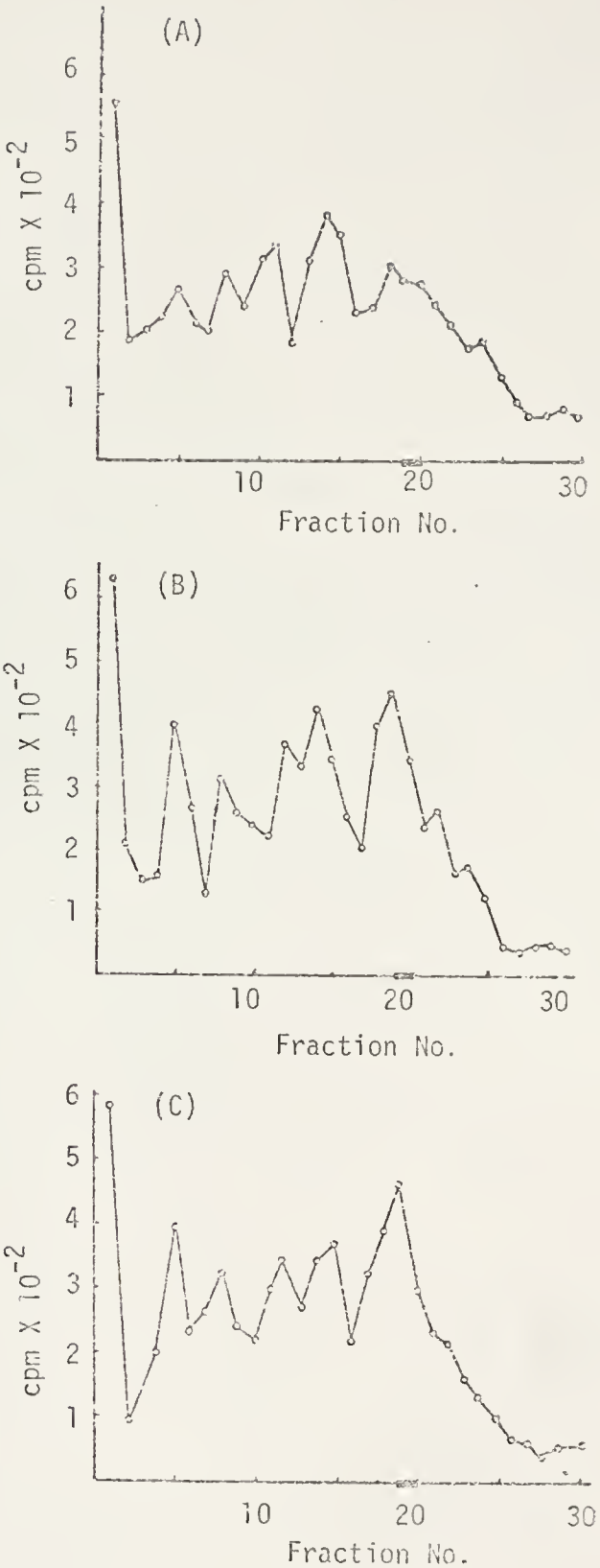
Conditions for amino acid incorporation and immunoprecipitation have been described in Materials and Methods. Each value represents the average of 3 determinations.

Embryo age (days)	Polysome	Albumin cpm	Total TCA insoluble polypeptides cpm	Albumin as % of total TCA insoluble polypeptides
12	free	655	116890	0.56
	bound	4862	107380	4.52
16	free	564	129340	0.44
	bound	4148	98570	4.21
19	bound	3760	86830	4.33

A cell-free system composed of low speed supernatant of liver homogenate seemed to be suitable for testing this possibility for the following reasons. The low speed supernatant contains both membrane-bound and free polysomes present in their *in vivo* ratio. This system has been shown to synthesize polypeptides with an *in vivo*-like pattern (Hill, Wilson and Hoagland, 1972). Another advantage of this system is that it requires only small liver samples which allows the study of early embryos.

In the following experiments, the cell-free reaction products synthesized by 3,000 X g supernatant derived from 9-day, 13-day and 16-day embryos were compared. After incubation for protein synthesis, the reaction mixtures were centrifuged to sediment microsomes. The supernatants of 9-day, 13-day and 16-day reaction mixtures so prepared contained 35%, 23% and 17% of the total hot trichloroacetic acid insoluble counts respectively. This is probably due to the presence of different amounts of membrane-bound polysomes in the samples, because a lower percentage of radioactivity is released from membrane-bound polysomes as shown in the previous experiment and in the results of other workers (Andrews and Tata, 1971). The supernatants did not contain albumin as judged by gel electrophoresis and immunoprecipitation (not shown). The microsomal pellets were frozen, thawed and sonicated. The sonic supernatants were dialyzed and analyzed by gel electrophoresis. The gel patterns are shown in Figure 29. In each sample, a radioactive peak comigrated with standard albumin. The amount of albumin synthesized in each sample was again estimated by counting the immunoprecipate after reaction with anitserum against albumin. The relative proportion of albumin synthesized by 9-day embryos was much less than that synthesized

Figure 29. Polyacrylamide gel electrophoresis of cell-free protein products synthesized by 3,000 X g supernatant from livers at various stages of development. Conditions for amino acid incorporation and analysis of products have been described in Materials and Methods. About 7,000 to 9,000 cpm of trichloroacetic acid insoluble material was applied to each gel. The protein staining band of standard albumin is indicated in the graph. (A) 9-day embryos (B) 13-day embryos (C) 16-day embryos.


(A)
cpm X 10^{-2}
6
5
4
3
2
1
10
20
30
Fraction No.
(B)
cpm X 10^{-2}
6
5
4
3
2
1
10
20
30
Fraction No.
(C)
cpm X 10^{-2}
6
5
4
3
2
1
10
20
30
Fraction No.

by 13-day and 16-day embryos (Table 10). The increase in the amount of albumin synthesis with developmental age agrees with the increase in albumin concentration in the serum. This result confirms the previously postulated correlation between an increase in the proportion of membrane-bound polysomes and an increase in specific protein synthesis.

Table 10

Albumin Synthesis by 3000 X g Supernatant Derived from Embryos of Various Ages

Conditions for amino acid incorporation and immunoprecipitation have been described in Materials and Methods. Each value represents the average of 3 determinations.

Experiment Number	Embryo age (days)	Albumin cpm	Total TCA insoluble polypeptides cpm	Albumin as % of total TCA insoluble polypeptides
1	9	794	72320	1.1
	13	2174	90040	2.4
	16	2894	82390	3.5
2	9	403	44760	0.9
	16	1379	47690	2.9

CONCLUSIONS

The embryological development of the liver involves at least two major processes, the increase in the size of the liver, which represents increases in the size and number of cells, and the development of the functional differentiation in the liver cells. In terms of protein synthesis, the former process must be dependent on the synthesis of intracellular, *i.e.* structural or constituitive proteins, the latter process involves the synthesis of proteins which are secreted for function in other portions of the organism.

In the domestic fowl, the liver grows most rapidly in size from the 5th to the 7th day of embryonic development; thereafter the growth rate gradually decreases (Romanoff, 1967, p. 267). On the other hand, the liver begins to synthesize albumin at the 8th day of development, and the concentration of albumin in the embryonic blood increases rapidly with increasing age (Weller and Schechtman, 1962; Zaccheo and Grossi, 1967). Therefore, as development progresses, it is apparent that the liver cells gradually undergo a quantitative shift from the biosynthesis of intracellular proteins to that of secreted proteins.

Changes in the protein synthesizing activity of the chick liver during development have been studied using liver slices (Duck-Chong, Pollak and North, 1964). Incorporation of labeled amino acid into liver slices was found to be more rapid in the younger embryos. However, changes in the protein synthesizing activity of cell-free systems and

the activity of components of the cell-free system during chick embryo development have not been studied previously.

Our results indicate that the amino acid incorporating activity of whole cell sap decreases with increasing embryo age, whereas the activity of the total polysome population remains constant with age. It would appear that some component or components in the cell sap control the rate of general protein synthesis. Of the individual activities we examined, we found that the cell sap aminoacyl-tRNA synthetase activity remained constant, whereas the activity in transferring amino acid into polypeptide decreased with embryo age. Thus the decline in overall cell sap amino acid incorporating activity may be ascribed to some component involved in a stage of protein synthesis following the aminoacylation of the tRNAs. The increase in cell sap ribonuclease activity with age which we observed is one possibility. Changes in the activity of initiation and elongation factors present in the cell sap are the other possibilities.

We found that the amount of total polysomes remained constant with age, but the average length of total polysomes decreased with increasing embryo age. This is consistent with increases in the synthesis of albumin, since albumin is among the smaller proteins elaborated by the liver. The possibility remains that the decrease in polysome length we described may be an experimental artifact caused by the presence of higher ribonuclease activity in livers from older embryos, although the polysomes were all isolated in the presence of nuclease inhibitors.

Our results indicate that the amino acid incorporating activity of membrane-bound polysomes decreases with increasing embryo age. One

possible explanation is the presence of more nuclease activity in the polysomes from older embryos. Since reinitiation in the cell-free system is limited, shorter polysome length would result in lower overall amino acid incorporation. Thus the decrease in amino acid incorporation may be caused by a lowering of the average length of membrane-bound polysomes with age.

We found that albumin synthesis per weight of membrane-bound polysomes remained constant, while a large proportion of polysomes were membrane-bound as development proceeded. Therefore, the rise in albumin concentration in the embryonic blood may result from even diminished albumin synthesis carried out by a larger complement of membrane-bound polysomes. Study of albumin synthesis in fetal and neonatal mouse liver has shown that the percentage of albumin synthesis by membrane-bound polysomes remained constant during development (Tamaoki, Thomas and Schindler, 1974; Koga and Tamaoki, 1974). However, the increase in albumin concentration in the blood was ascribed to a net increase in the number of albumin synthesizing polysomes with the growth of the liver.

A net rise in circulating protein concentration may be the result of increased protein synthesis, or decreased protein degradation, or both. Our results indicate that the increase in albumin concentration in the blood is at least partly the result of increased albumin synthesis. However, the catabolism of albumin in chick embryos has not been studied so far, and would be an interesting subject for further investigation.

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BIOGRAPHICAL SKETCH

Mon-Li Hsiung Chu was born on July 27, 1948, in Kwangtung, China and raised in Taipei, Taiwan. After graduation from Taipei First Girls' High School in 1966, she entered the National Taiwan University where she majored in Agricultural Chemistry. She received the degree of Bachelor of Science in June, 1970.

She entered the graduate school of the University of Florida in September, 1970. Since then, she has pursued her work toward the degree of Doctor of Philosophy in the Department of Biochemistry.

She was married to Shaw-Chang Chu in December, 1972.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Melvin Fried, Chairman
Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard P. Boyce
Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rusty D. Mans
Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Carlo Moscovici
Professor of Pathology

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Dean, Graduate School

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